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TITLE OF INVENTION

SYSTEM FOR REGULATION OF EXPRESSION USING PPAR NUCLEAR RECEPTORS

APPLICANT(S) FOR DO/EO/US

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Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a FIRST submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a SECOND or SUBSEQUENT submission of items concerning a filing under 35 U.S.C. 371.
3. [ ] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] has been transmitted by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US)
6. [X] A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [X] have been transmitted by the International Bureau.
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   d. [ ] have not been made and will not be made.
8. [X] A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [X] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)), and Power of Attorney (unexecuted).
10. [X] A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. Below concern other document(s) or information included:

11. [ ] An information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [ ] A FIRST preliminary amendment.
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CERTIFICATION UNDER 37 CFR 1.10

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I hereby certify that this paper (along with any referred to as being attached or enclosed) is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 CFR 1.10 on the date indicated Above and is addressed to Box PCT, Commissioner for Patents, Washington, DC 20231, Attn. EO/US

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17. [X] The following fees are submitted: Basic National Fee (37 CFR 1.492(a)(1)-(5): Search Report has been prepared by the EPO or JPO.....$ 860.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) .....$ 720.00 No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))..$ 790.00 Neither international preliminary examination fee (37 CFR 1.482) nor International search fee (37 CFR 1.445(a)(2)) paid USPTO.....$1070.00 International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4).....$ 98.00				CALCULATIONS	PTO use only
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Independent Claims	- 3 =		X $ 80.00	$ 0.00	
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Date: December 18, 2001

SYSTEM FOR REGULATION OF EXPRESSION USING PPAR NUCLEAR RECEPTORS

The present invention relates to the field of biology. It relates in particular to the field of the regulation of the expression of genes and, more particularly, it describes the design and development of a new system for the pharmacological regulation of the expression of transgenes. The invention is based in particular on the use of constructs of human origin for activating the transcription of the transgene. The invention thus describes new compositions, constructs and methods allowing the effective regulation of the expression of a nucleic acid in vitro, ex vivo or in vivo, for example in muscle cells. The applications which result from the present invention are many, in the experimental, clinical, therapeutic or diagnostic fields, for example.

Controlling the level and the duration of the expression of transgenes is necessary for many applications. Thus, in gene therapy, the success of the therapy may require a specific assay of the protein synthesized from the transgene. Likewise, the production of recombinant proteins in vitro may be improved using inducible expression systems, allowing for example uncoupling of the growth and production phases. The construction of transgenic animals, the study of the effects of a gene and of the

bioavailability of a protein, and the like, are so many situations in which an appropriate control of genetic expression may be used and may provide improvement.

Various artificial regulators of transcription have been designed in the prior art, which are activated by a xenobiotic molecule which bind to the promoter sequences for transcription of the transgene.

A first illustration of these regulators was constructed by fusion of the E. coli Lac repressor with the herpes simplex virus (HSV) VP16 transactivator domain. Two versions of these regulators exist, one capable of being activated by isopropyl β-D-thiogalactoside (IPTG) and the other inactivated by IPTG (Baim S. et al., *Proc Natl Acad Sci USA,* **88** (1991) 5072-5076; Labow M. et al., *Mol. Cell. Biol.,* **10** (1990) 3343-3356).

Another system was constructed by fusion of the E. coli Tet repressor with the HSV VP16 transactivator domain. Two versions of these regulators also exist, one capable of being activated by tetracycline or its derivatives and the other inactivated by these same molecules (Gossen M. and Bujard H., *Proc Natl Acad Sci USA,* **89** (1992) 5547-5551; Gossen M. et al., *Science,* **286** (1995) 1766-1769).

Another system was constructed by fusion of the DNA-binding domain of the S. cerevisiae GAL4

protein with the ligand-binding domain of the human progesterone receptor and the HSV VP16 transactivator domain; this version is activated by a progesterone analogue such as RU486 (Wang Y. et al., *Proc Natl Acad Sci USA*, **91** (1994) 8180-8184). A fusion of the drosophila ecdysone receptor with the HSV VP16 transactivator domain has also been described, activated by ecdysone and the analogues of this steroid hormone (No D et al., *Proc Natl Acad Sci USA*, **93** (1996) 3346-3351). Another system takes advantage of the capacity of certain immunosuppresive molecules (cyclosporin A, rapamycin and its derivatives) to promote the combination of certain cellular proteins. A transcriptional regulator then consists of two protein subunits; the first may be formed by the fusion of a chimeric DNA-binding domain and of three copies of the human FKBP protein and the second by the fusion of the rapamycin-binding domain of the human FRAP protein and of the transactivator domain of the human NFkB p65 subunit. This transcriptional regulator is activated by rapamycin which allows the dimerization of the two subunits (Rivera V. et al., *Nat. Med.*, **2** (1996) 1028-1032).

Although these systems make it possible to obtain satisfactory levels of regulation in some tissues, they exhibit, nevertheless, certain disadvantages which limit their conditions of use.

Thus, these transcriptional regulators are xenogenic proteins in humans. They indeed consist of protein fragments obtained from bacteria, viruses, yeasts or insects or, when the protein domains are of human origin, their joining creates sequences which are foreign to humans. These protein domains may therefore induce a cytotoxic immune reaction, causing the destruction of the cells which express the gene of interest under the control of the xenogenic transcriptional regulator, and thus the termination of the expression of the transgene. This situation may necessitate the use of repeated administrations of the therapeutic gene, which constitutes a major disadvantage, in particular when this involves a traumatic surgical act, and which is not always effective, in particular when the vector of the therapeutic gene is a virus whose first injection causes an immune reaction. In addition, the levels of expression observed with the prior art systems of regulation are not always satisfactory.

The need therefore exists for an improved system of regulating expression, compatible with use in vivo, which can be used in various tissues, and which ensures high levels of expression in the activated state. The present invention provides a solution to these problems.

The present invention indeed relates to a system of regulation using an activator of human origin. This should make it possible to avoid the repeated administrations of the therapeutic gene.

The present invention describes in particular an improved system of inducible expression using the PPAR (Peroxisome proliferator-activated receptors) nuclear receptors as transcriptional regulators. The use of PPRE in a hepatospecific expression system has been described in application WO 98/21349. The improved system according to the invention currently makes it possible to produce the transcriptional regulator (a PPAR protein of human origin, and therefore essentially nonxenogenic in humans) and the inducible promoter, which controls the expression of the transgene, is composed, on the one hand, of a minimum promoter and, on the other hand, of a PPAR response element (PPRE). The system of the invention is activable, in vitro and also in vivo, in particular in the muscle, by the ligands specific for the PPARs. Furthermore, the level of expression of the transgene, obtained after activation, is comparable to that of a strong promoter such as the hCMV-IE promoter.

The system according to the present invention therefore exhibits numerous advantages, simultaneously in terms of substantial induction, of tolerance (in

particular for an in vivo use), of strength and of conditions of use.

The invention therefore describes new constructs for the preparation and the use of this system, in particular promoter regions, expression cassettes and plasmids. The invention also describes new PPAR constructs allowing an improved control of the expression of genes, as well as combinations of these different constructs. The invention shows, in addition, that these methods and compositions allow substantial control and regulation of the expression in vitro and in vivo. The invention also relates to cells comprising constructs of the invention, as well as methods for screening compounds which are ligands for PPARs, for example.

More particularly, a first subject of the invention consists in a composition comprising:

(a) a first element comprising a nucleic acid of interest under the control of an inducible promoter comprising a PPAR response element and a minimal transcriptional promoter, and

(b) a second element comprising a nucleic acid encoding a PPAR under the control of a transcriptional promoter,

for their use simultaneously, separately or spaced out over time.

In a more specific mode, the compositions of the invention comprise in addition:

(c) a ligand for PPAR,

also for a use simultaneously, separately or spaced out over time.

Advantageously, they comprise, in addition, an element (d) comprising a nucleic acid encoding a retinoid X recepetor (RXR) under the control of a transcriptional promoter.

The expression for a use simultaneously, separately or spaced out over time indicates that the elements (a), (b) and, where appropriate, (c) and/or (d) may be prepared separately, packaged separately, and used sequentially, to allow control of the expression of the nucleic acid of interest. Typically, the elements (a) and (b), and optionally (d) are prepared and packaged together, whereas the compound (c) is packaged separately and used spaced out over time with (a) and (b), and optionally (d), the combination of these different elements in a cell, a tissue, an organ and the like leading to the desired effect of regulation of expression.

In this regard, in a specific embodiment of a composition of the invention, the elements (a), (b) and optionally (d) are carried by distinct genetic constructs.

In another specific and preferred embodiment of a composition of the invention, the elements (a), (b) and optionally (d) are assembled in the same genetic construct. The present invention thus describes complex genetic constructs allowing the expression of a product of interest and of a PPAR. These constructs are particularly advantageous since they contain, on their own, all the genetic elements necessary for the regulated expression of the nucleic acid of interest.

The genetic construct(s) may be of a varied, in particular plasmid, episomal, chromosomal, viral or phage, nature and/or origin, and the like. Preferably, the genetic construct is a plasmid or viral vector.

By way of illustration of plasmids separately carrying the elements (a) or (b), there may be mentioned for example the plasmids JxnS-TK-pGL3, JxnAS-TK-pGL3, DR1xnS-TK-pGL3, DR1xnAS-TK-pGL3, JxnAS-CMV-pGL3, pSG5-hPPARg2g2 or Jx10AS-CMV-EF-pGL3, which will be described in detail later.

By way of illustration of plasmids in which the elements (a) and (b) have been assembled, there may be mentioned for example the plasmids Jx5AS-TK-Luc-hPPARg2, SV-g2-J10-C-pGL3, hPPARg2-CMV-Jx5AS-TK-pGL3 or hPPARg2-CMV-Jx10AS-CMV-pGL3, which will be described in detail later.

As an example of a viral vector, there may be mentioned in particular a recombinant adenovirus, a

recombinant retrovirus, an AAV, a herpesvirus, a vaccinia virus and the like, whose preparation may be carried out according to methods known to persons skilled in the art.

The arrangement and the structure of the genetic constructs will be described in greater detail in the text which follows.

In this regard, as indicated above, the element (a) comprises an inducible promoter comprising at least:

- a PPAR response element, and
- a minimal transcriptional promoter.

A PPAR response element (PPRE, "Peroxysome Proliferator Response Element") is a nucleic acid region capable of binding a PPAR, it being possible for the binding of the PPAR to then mediate a signal around neighbouring nucleic regions. A PPAR response element is therefore a nucleic acid region capable of binding PPARs. For carrying out the invention, the PPAR response element comprises more particularly one or more PPAR-binding sites. Such binding sites have been described in the prior art, such as for example, in different human promoters (gene for apolipoprotein AII, for example). Such sites may also be artificially constructed, and tested for their PPRE properties, as is described below.

In a specific embodiment, the PPAR response element comprises one or more sites having the sequence TCAACCTTTACCCTGGTAG (SEQ ID NO:1) or functional variants of this sequence. The sequence SEQ ID NO:1 corresponds to the J region of the human apoAII promoter (nucleotides -734 to -716).

In another specific embodiment, the PPAR response element comprises one or more sites having the sequence AGGTCAAAGGTCA (SEQ ID NO:5) or functional variants of this sequence. The sequence SEQ ID NO:5 corresponds to the consensus region DR1.

The term functional variant designates any modified sequence conserving the properties of PPRE as mentioned above, that is to say in particular the capacity to bind a PPAR. The modifications may comprise one or more additions, mutations, deletions and/or substitutions of nucleotides in the sequence considered. These modifications may be introduced by conventional molecular biology methods, such as in particular site-directed mutagenesis or, more practically, by artifical synthesis of the sequence in a synthesizer. Generally, the variants conserve at least 50% of the residues of the initial sequence indicated. More preferably, the variants possess modifications affecting less than 5 nucleotides in the sequence considered. The variants thus obtained are

then tested for their PPRE activity. This property may be checked in various ways, and in particular:

(i) by bringing the test sequence into contact with a PPAR and a retinoid X receptor (RXR), preferably in an acellular test, and detecting the formation of a complex (for example by gel migration retardation);

(ii) by inserting the test sequence into an expression cassette comprising a minimal promoter and a reporter gene, introducing the cassette into a cell, and detecting (where appropriate assaying) the expression of the reporter gene in the presence and in the absence of a PPAR and of a ligand for a PPAR;

(iii) by any other technique known to a person skilled in the art, making it possible to detect the interaction between a nucleic acid and a protein, for example.

A variant is considered to be functional for the purpose of the present invention when the activity measured, for example in (ii) above, is preferably at least equal to 50% of that measured with a site having the sequence SEQ ID NO:1 or 5, more preferably at least equal to 75%. Functional variants of PPAR-binding sites for the purpose of the invention are described, for example, in Juge-Aubry et al., (J. Biol. Chem. 272 (1997) 25252) and in Nakshatri et al. (NAR 26 (1998)

2491), which are incorporated into the present application by way of reference.

The retinoid X receptors (RXR) are encoded by three genes RXRα, RXRβ and RXRγ, whose isolation and sequence have been described (Mangelsdorf DJ et al. (1990), *Nature* **345**, 224-229; Mangelsdorf DJ et al. (1992), *Genes Dev* **6**, 329-344). Preferably, the element (d) encodes the human RXRα.

As regards the PPAR/RXR heterodimerization, two reviews may be consulted: Mangelsdorf DJ and Evans RM (1995), *Cell* **83**, 841-850 and Wilson TM and Wahli W (1997), *Current Opinion in Chemical Biology* **1**, 235-241. The article by Schulman IG et al. (1989), *Molecular and Cellular Biology* **18**, 3483-3494 describes transactivation by the PPARγ/RXRα heterodimer.

The use of the element (d) is capable of synergizing the activity of the element (b).

As indicated above, in the compositions according to the invention, the PPAR response element may comprise several sites for binding to a PPAR. This may be a repetition of the same site, or combinations of different sites, the repetition of identical sites being preferred. More particularly, the response element comprises up to 30 binding sites, preferably from 3 to 20, more preferably from 5 to 15. A preferred embodiment of the invention is a construct comprising from 10 to 15 binding sites, the results presented in

the examples indeed show the advantageous properties of such constructs in terms of induction and of level of expression, in particular in the muscle cells.

For the preparation of an inducible promoter according to element (a) of the compositions of the invention, the PPAR response element is combined with a transcriptional minimal promoter. The minimal promoter is a transcriptional promoter having a basal activity which is low or inexistant, and capable of being increased in the presence of a transcriptional activator (interaction of a PPAR activated with the PPRE element). A minimal promoter may therefore be a promoter which is naturally weak in mammalian cells, that is to say which produces a nontoxic and/or an insufficient expression in order to obtain a pronounced biological effect. Advantageously, a minimal promoter is a construct prepared from a native promoter, by deletion of a region or regions not essential for the transcriptional activity. Thus, this is preferably a promoter comprising essentially a TATA box, generally of less than 160 nucleotides in size, centred around the codon for initiation of transcription. A minimal promoter can thus be prepared from strong or weak viral or cellular promoters such as for example the promoter of the herpesvirus thymidine kinase (TK) gene, the CMV immediate promoter, the PGK promoter, the promoter of the muscle creatine kinase (MCK) gene, the promoters of

the genes for the various skeletal muscle actin isoforms, the promoter of the desmin gene, the promoter of the vimentin gene, the promoters of the myosin light chain or heavy chain genes, and the like. Specific examples of minimum promoters are represented by nucleotides -54 to +48 of the CMV or -105 to +56 of the TK promoter, for example. It is understood that any variant of these promoters or similar constructs from other promoters may be constructed by persons skilled in the art and used within the framework of the present invention.

The minimal promoter (Pmin), the PPAR response element (PPRE) and the nucleic acid of interest (NA) are arranged functionally in the element (a), that is to say such that the minimal promoter controls the expression of the nucleic acid of interest and that its activity is regulated by the PPRE element. Generally, these regions are therefore arranged in the following order, in the 5'→3' orientation: PPRE-Pmin-NA. However, any other functional arrangement can be envisaged by persons skilled in the art without departing from the present invention.

In addition, the various functional domains above may be directly linked to each other, or separated by nucleotides which do not significantly affect the regulated character of the promoter of the element (a). Such nucleotides may be neutral residues

from the functional point of view, resulting, for example, from cloning steps (PCR ends, restriction sites, and the like). These nucleotides may also possess biological properties which make it possible to confer improved characteristics or performances to the system of the invention (enhancer of housekeeping genes, tissue specific enhancer, silencer, intron, splicing site, and the like). In this regard, in a specific embodiment of the invention, the inducible promoter comprises, in addition, an enhancer region. Such a region advantageously makes it possible to increase the levels of expression of the nucleic acid of interest. Such an enhancer (E) region is preferably positioned in 3' of the minimal promoter, between the latter and the nucleic acid of interest, according to the following scheme (5'→3'): PPRE-Pmin-E-NA.

Moreover, in the constructs of the invention, the minimal promoter and the PPAR response element may be present either in the same orientation (that is to say in the direction of transcription), or in the opposite orientation (that is to say that the PPAR response element is in the antisense orientation relative to transcription by the Pmin promoter). As illustrated in the examples, these two embodiments allow an effective control of the regulation of the expression in vitro and in vivo.

As indicated above, the element (b) of the compositions according to the invention comprises at least:

- a nucleic acid encoding a PPAR,
- under the control of a second transcriptional promoter.

The PPARs belong to the superfamily of nuclear hormone receptors, and are grouped into three distinct groups, PPARα, PPARδ (also called NUC-1 or PPARβ) and PPARγ. The isolation and the sequence of many human PPARs have been described in the literature (see in particular Sher T. et al., *Biochemistry,* **32** (1993) 5598-5604; Mukherjee R. et al., *J. Steroid Biochem. Molec. Biol.*, **51** (1994) 157-166; Fajas L. et al., *J. Biol. Chem.*, **272** (1997) 18779-18789; Mukherjee R. et al., *J. Biol. Chem.*, **272** (1997) 8071-8076; Schmidt A. et al., *Mol. Endocrinol.* **6** (1992) 1634-1641). The PPARγ promoter has, in addition, been recently cloned, as described by application WO 99/05161.

In a preferred embodiment of the invention, the nucleic acid encoding a PPAR encodes a human PPAR, in particular a PPARα or a PPARγ. The results presented in the examples indeed show that the use of these molecules ensures for the system of the invention high levels of regulation and expression, in particular in the muscle cells.

According to a first embodiment, this is a PPARα or a PPARγ in its native form, that is to say without modification of primary structure relative to the natural molecule.

According to another embodiment, this is a modified PPAR comprising several ligand-binding sites.

In this regard, the present invention describes and also has as subject any modified PPAR comprising several ligand-binding sites. More particularly, this is a PPARα or a PPARγ, still more preferably a PPARγ. Preferably, the modified PPARs according to the invention comprise from 2 to 5 ligand-binding sites, more preferably from 2 to 4 binding sites. This is more particularly PPAR containing 2 to 5 copies of the E and F domains involved in the binding to the ligand. The PPAR proteins contain different domains: the N-terminal A/B domain which contains a transactivating region not dependent on the ligand, the C domain which is the DNA-binding domain (DBD) and the D domain which is a hinge region, and the E/F domains which contain a transactivating region dependent on the ligand. The E/F domains are also called ligand-binding domain (LBD) (see in particular Schoonjans K. et al., *Biochim. Biophys. Acta,* **1302** (1996) 93-109). The limits of the E/F domains vary from one PPAR to another. By way of example, for the human PPARγ2 isoform used, the E/F domain extends from amino acid 284 to amino acid

505. The present invention now shows that it is possible to construct modified PPARs comprising several repeated E and F domains, and that these modified PPARs are functional and possess improved properties of inducibility by the ligands for the PPARs. Such constructs therefore represent an embodiment and a specific subject of the present invention.

A typical example of modified PPAR according to the invention is a PPARγ containing 2 ligand-binding sites (that is to say two E and F domains). The complete protein sequence of PPARγ2γ2 is represented on the sequence SEQ ID NO:24.

SEQ ID NO:24

MGETLGDSPIDPESDSFTDTLSANISQEMTMVDTEMPFWPTNFGISSVDLSVMEDHSHSFDI
KPFTTVDFSSISTPHYEDIPFTRTDPVVADYKYDLKLQEYQSAIKVEPASPPYYSEKTQLYN
KPHEEPSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIYDRCDLNCRIHKKS
RNKCQYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQLNPESADLRALAKHLYD
SYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIF
QGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEIIYTMLASLMNKDGVLISEG
QGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPI
EDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPL
LQEIYKDLYAWAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGC
QFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEIIYTMLASLMNKDGVLISEGQGF
MTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPIEDI
QDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPLLQE
IYKDLY

The invention also relates to any variant of the sequence SEQ ID NO:24 conserving a PPAR-type activity (the capacity to activate, in the presence of a PPAR ligand such as BRL49653, a promoter containing a PPRE sequence). The variants are understood to mean any mutant, deletant and/or polypeptide containing one or more additional residues. Preferably a variant

conserving at least 80% of the residues of the sequence ID NO:24.

In addition, the invention also relates to any nucleic acid encoding such a modified PPAR. This may be a DNA (in particular a cDNA or a synthetic or semisynthetic DNA) or an RNA. This DNA may be constructed according to conventional molecular biology methods known to persons skilled in the art (synthesis, ligations, screening of libraries and the like). It is advantageously any nucleic acid comprising a sequence encoding a polypeptide having the sequence SEQ ID NO:24, or hybridizing with a sequence encoding a polypeptide of SEQ ID NO:24, and encoding a polypeptide with a PPAR-type activity. In addition, this DNA may comprise a transcriptional promoter and/or terminator, for example.

The second transcriptional promoter, controlling the expression of the nucleic acid encoding the PPAR, may be any strong or weak, ubiquitous or selective, constitutive or regulated promoter which is functional in mammalian cells, in particular in human cells. This may be a domestic cellular promoter (i.e., a mammalian, in particular a human, gene), a natural or synthetic, simple or complex, viral, bacterial, insect or plant promoter, and the like. Examples of appropriate promoters for this element (b) are in particular viral promoters (SV40 virus immediate

promoter, CMV virus immediate promoter, retrovirus LTR, herpesvirus TK promoter) or cellular promoters (PGK, albumin or EF1α promoter, or promoter of genes which are highly expressed in the muscle such as: promoter of the muscle creatine kinase (MCK) gene, promoters of the genes of the various skeletal muscle actin isoforms, promoter of the desmin gene, promoter of the vimentin gene, promoters of the myosin light chain or heavy chain genes). Moreover, the promoter may be modified by introduction of one or more enhancer regions, such as the enhancer region of intron 2 of the beta-globin gene, enhancer of the CMV virus very early gene, EF1α enhancer, silencer region(s), regions conferring a tissue specificity (for example regions isolated from tissue-specific promoters such as: promoter of the muscle creatine kinase (MCK) gene, promoters of the genes of the various skeletal muscle actin isoforms, promoter of the desmin gene, promoter of the vimentin gene, promoters of the myosin light chain or heavy chain genes) or a regulable character, or by deletion of regions not essential to the activity, for example. Such promoters may be used to express the RXR, contained in the element (d).

Preferred examples of a second promoter are the viral promoters, in particular the SV40 virus early promoter and the CMV immediate promoter, or derivatives thereof.

Moreover, in a specific embodiment, when the elements (a) and (b), and optionally (d), are assembled in the same genetic construct, the second transcriptional promoter (of the element (b)) and the inducible promoter of the element (a), and optionally the promoter of the element (d), may be grouped so as to form only one common, in particular bidirectional, promoter region, as will be explained in detail in the text which follows.

To this effect, another subject of the present invention consists in a vector comprising an element (a) and an element (b), and optionally an element (d), as defined above.

According to a first variant of the invention, the elements (a) and (b), and optionally (d), are in the same orientation in the vector. Such a variant is illustrated for example by the plasmid SV-g2-J10-C-pGL3 (Figure 17).

According to another variant of the invention, the elements (a) and (b), and optionally (d), are in the opposite orientation in the vector. Such a variant is illustrated for example by the plasmids represented in Figures 16, 18 and 19. More preferably, in this variant embodiment, the inducible promoter of the element (a) and the transcriptional promoter of the element (b) are assembled in the vector to form a regulable bidirectional promoter. Such an

embodiment is illustrated for example by the plasmids represented in Figures 18 and 19.

In this regard, a specific subject of the invention consists in a vector characterized in that it comprises, in the 5'→3' direction, a first nucleic acid encoding a PPAR, a first minimal transcriptional promoter controlling the expression of the said first nucleic acid, one or more PPAR response element(s), a second minimal transcriptional promoter and, under the control of the said second minimal transcriptional promoter, a second nucleic acid encoding a product of interest.

This type of construct is advantageous since it allows the co-expression of the two nucleic acids in the same plasmid, and the amplification of this expression by the regulation of the two nucleic acids by the PPARs and their ligands.

The expression of the nucleic acid of interest in the compositions of the invention is generally activated in the presence of a PPAR ligand (element (c)). In this regard, according to the PPAR used, various types of ligands, natural or synthetic, may be used.

Thus, the PPARα-activating ligands are for example the fibrates such as fibric acid and its analogues. As analogues of fibric acid, there may be mentioned in particular gemfibrozyl (Atherosclerosis

114(1) (1995) 61), bezafibrate (Hepatology 21 (1995) 1025), ciprofibrate (BCE&M 9(4) (1995) 825), clofibrate (Drug Safety 11 (1994) 301), fenofibrate (Fenofibrate Monograph, Oxford Clinical Communications, 1995), clinofibrate (Kidney International. 44(6) (1993) 1352), pirinixic acid (Wy-14,643) or 5,8,11,14-eicosatetranoic acid (ETYA). These various compounds are compatible with a biological and/or pharmacological use in vitro or in vivo.

The PPARγ-activating ligands may be chosen from natural and synthetic ligands. As natural ligands, there may be mentioned fatty acids and eicosanoids (for example linoleic acid, linolenic acid, 9-HODE, 5-HODE) and as synthetic ligands, there may be mentioned thiazolidinediones, such as in particular rosiglitazone (BRL49653), pioglitazone or troglitazone (see for example Krey G. et al., *Mol. Endocrinol.*, **11** (1997) 779-791 or Kliewer S. and Willson T., *Curr. Opin. in Gen. Dev.* **8** (1998) 576-581) or the compound RG12525.

Moreover, the compositions according to the invention may contain several PPAR activators in combination, and in particular a fibrate or a fibrate analogue combined with a retinoid.

The subject of the invention is also the use of a composition or of a vector as defined above for expressing a nucleic acid of interest in a cell ex vivo or in vitro.

In this regard, the nucleic acid may be any nucleic acid (DNA, RNA) encoding a product of interest (RNA, protein, polypeptide, peptide and the like). It may be a product of interest in the food, therapeutic or vaccine sector, a marker, and the like.

The invention also relates to the use of a composition or of a vector as defined above for the preparation of a product intended for expressing a nucleic acid of interest in a cell in vivo.

The subject of the invention is also a method for the regulated expression of a nucleic acid in a cell, comprising bringing the said cell into contact with a composition or a vector as defined above.

For a use in vitro or ex vivo, the cells may be brought into contact with the compositions or vectors of the invention according to various protocols. Thus, the cells in culture may be incubated directly with the elements (a), (b) and (c), and optionally (d), of the invention, for example with a vector containing the elements (a) and (b) and in the presence of the ligand (c). Alternatively, the cells may be incubated in a first instance with the elements (a) and (b) and optionally (d) (in particular assembled in the same vector) and then, in a second instance (after culture and optionally selection of the modified cells), the element (c) may be added. This latter type of protocol makes it possible, for example, to uncouple

the culture phase (or the cell expansion phase) from the nucleic acid expression phase. These experiments may be carried out in any appropriate medium and device, preferably in a plate, dish, flask, in the sterile condition. The quantities of cells, vector and ligand can be easily adapted by persons skilled in the art, on the basis of the information provided in the examples and of their general knowledge.

For a use in vivo, the cells (or organs, tissue, and the like) are brought into contact, by administration of the elements (a), (b) and (c), and optionally (d), in vivo, simultaneously, separately or spaced out over time. To this effect, the elements (a) and (b), and optionally (d), optionally in the form of a single genetic construct, are generally administered by the parenteral, in particular intramuscular, intravenous, subcutaneous, intradermal, intratumoral or stereotaxic route. The choice of the mode of adminstration may be guided by the application envisaged, the tissue targeted and/or the type of product of interest encoded by the transgene. For this administration, the compositions of the invention may comprise any agent promoting cellular transfection (cationic polymer, lipid and the like). In a specific mode, the compositions are administered by the intramuscular route, and the genetic constructs are

used in the form of a "naked" nucleic acid, that is to say without added transfection agent.

Likewise, when the elements (a) and (b), and optionally (d), are introduced by means of viral vectors, no additional transfection agent is necessary.

As illustrated in the examples, the ligand (c) may be administered before, simultaneously or after the elements (a) and (b), and optionally (d).

In this regard, the administration of the ligand may be carried out by the oral, anal, intravenous, intraperitoneal or intramuscular route, for example.

The doses used may be adapted by persons skilled in the art, on the basis of the in vivo data published in the literature. Thus for example, for a form not soluble in water, typical doses of ligand such as BRL49653 are between 5 and 50 mg/kg, for example 30 mg/kg, which make it possible to obtain a plasma concentration close to about 15 µg/ml at least. For a water-soluble form of ligand, whose bioavailability is greater (for example a maleate salt of BRL49653), the typical doses are lower, generally less than 5 mg/kg, for example from 0.01 to 1 mg/kg. These doses can be quite obviously adapted by persons skilled in the art as a function of the constructs used, the ligands used, and the desired applications and effects. In general, the results presented in the examples advantageously

show that the compositions of the invention make it possible to obtain in vivo a high and regulated expression, at ligand doses less than those normally used. In addition, although repeated administrations of ligand may be carried out, the results presented also show that the expression is high after a single dose of ligand.

Generally, the vector doses used may vary between 0.01 and 1000 μg, or more, depending on the desired applications.

The invention may be used for expressing a gene in various types of cells, tissues or organs, in vitro, ex vivo or in vivo. In particular, this may be a mammalian, preferably a human, cell, tissue or organ. By way of illustration, there may be mentioned muscle cells (or a muscle), hepatic cells (or the liver), cardiac cells (or the heart, the arterial or vascular wall), nerve cells (or the brain, the marrow and the like) or tumour cells (or a tumour).

Preferably, the constructs, compositions and method of the invention are used for the regulated expression of a nucleic acid in a muscle cell (or a muscle) in vitro, ex vivo or in vivo. The results presented in the examples illustrate more particularly the advantages of the invention in vivo or in vitro in this type of cells.

The invention also relates to any cell modified by bringing into contact with a composition or a vector as defined above.

The invention also relates to the use of a composition, of a vector or of a cell as defined above, in which the nucleic acid of interest is a reporter gene (such as for example secreted alkaline phosphatase or luciferase) for the screening in vitro, ex vivo or in vivo (in particular in the muscle cells or a muscle) for PPAR ligands. In this regard, the invention also describes a method for identifying PPAR ligands comprising the bringing into contact of a cell as defined above with a test molecule (or composition), and the detection of the expression of the nucleic acid of interest (the latter being preferably a reporter gene). The expression may, in addition, be compared with that observed in the absence of test compound or in the presence of a reference ligand, in order to evaluate the activity of the compound tested.

The invention also relates to the use of a composition or of a vector as defined above, for the construction of transgenic animals, in particular of nonhuman mammals, useful for preclinical studies, or for studies of bioavailability, or labelling, and the like.

The present invention will be described in greater detail with the aid of the examples which

follow and which should be considered as illustrative and nonlimiting.

LEGEND TO THE FIGURES

Figure 1 : Schematic representation of the plasmid FTKpGL3.

Figure 2 : Schematic representation of the plasmid Jx3S-TK-pGL3.

Figure 3 : Schematic representation of the plasmid Jx3AS-TK-pGL3.

Figure 4 : Schematic representation of the plasmid DR1x3S-TK-pGL3.

Figure 5 : Schematic representation of the plasmid DR1x3AS-TK-pGL3.

Figure 6 : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with : (i) 10 ng of plasmid FTKpGL3 (**a**), or Jx3S-TK-pGL3 (**b**), or Jx3AS-TK-pGL3 (**c**), or DR1x3S-TK-pGL3 (**d**), or DR1x3AS-TK-pGL3 (**e**), (ii) increasing quantities of plasmid pSG5-hPPARg2, and (iii) 20 ng of plasmid pRL-

null. The activity of each inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

Figure 7 : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with : (i) 10 ng of plasmid FTKpGL3 (**a**), or Jx3S-TK-pGL3 (**b**), or Jx3AS-TK-pGL3 (**c**), or DR1x3S-TK-pGL3 (**d**), or DR1x3AS-TK-pGL3 (**e**), (ii) increasing quantities of plasmid pSG5-hPPARa(Koz), and (iii) 20 ng of plasmid pRL-null. The activity of each inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

Figure 8 : Schematic representation of the plasmid Jx5AS-CMV-pGL3.

Figure 9 : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with : (i) 10 ng of plasmid Jx5AS-TK-pGL3 (**a**), or Jx5AS-CMV-pGL3 (**b**), (ii) increasing quantities of plasmid pSG5-hPPARg2, and (iii) 20 ng of plasmid pRL-null. The activity of each inducible promoter represents the

luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

Figure 10 : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with : (i) 10 ng of plasmid JxnAS-TK-pGL3, (ii) 10 ng of plasmid pSG5-hPPARg2, and (iii) 20 ng of plasmid pRL-null. The activity of each inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

Figure 11 : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with : (i) 10 ng of plasmid JxnAS-CMV-pGL3, (ii) 10 ng (**a**) or 50 ng (**b**) of plasmid pSG5-hPPARg2, and (iii) 20 ng of plasmid pRL-null. The activity of each inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

Figure 12 : Schematic representation of the plasmid pSG5-hPPARg2g2.

Figure 13 : Comparison of the transcriptional regulators hPPARg2 and hPPARg2g2. Mouse myoblasts (C2C12) are contransfected with: (i) 10 ng of plasmid Jx10AS-CMV-pGL3, (ii) increasing quantities of plasmid pSG5-hPPARg2 (**a**) or pSG5-hPPARg2g2 (**b**), and (iii) 20 ng of plasmid pRL-null. The activity of the inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase. (**c**) : factors for induction by BRL49653 obtained with the plasmid pSG5-hPPARg2 or the plasmid pSG5-hPPARg2g2. This induction factor is calculated by dividing the activity in the presence of BRL49653 by the activity in the presence of DMSO.

Figure 14 : Schematic representation of the plasmid Jx10AS-CMV-EF-pGL3.

Figure 15 : Activities of the inducible promoters evaluated in transient transfections in vitro in mouse myoblasts (C2C12). The cells are cotransfected with : (i) 10 ng of plasmid Jx10AS-CMV-pGL3 (**a**), or Jx10AS-CMV-EF-pGL3 (**b**), (ii) increasing quantities of plasmid pSG5-hPPARg2g2, and (iii) 20 ng of plasmid pRL-null. The activity of each inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

Figure 16 : Schematic representation of the plasmid Jx5AS-TK-luc-hPPARg2.

Figure 17 : Schematic representation of the plasmid SV-g2-J10-C-pGL3.

Figure 18 : Schematic representation of the plasmid hPPARg2-CMV-Jx5AS-TK-pGL3.

Figure 19 : Schematic representation of the plasmid hPPARg2-CMV-Jx10AS-CMV-pGL3.

Figure 20 : Comparison of the different versions of the system inducible in vitro. Mouse myoblasts (C2C12) are transfected with, for each version of the system, the same number of moles of inducible expression cassettes. The results are expressed as a percentage of the activity of the hCMV-IE promoter obtained using the plasmid pCMV-leadTK. The factors for induction with BRL49653 are calculated by dividing the activity in the presence of BRL49653 with the activity in the presence of DMSO. 1 = pSG5-hPPARg2 + Jx5AS-TK-pGL3; 2 = Jx5AS-TK-luc-hPPARg2; 3 = pSG5-hPPARg2g2 + Jx10AS-CMV-pGL3; 4 = pSG5-hPPARg2 + Jx10AS-CMV-pGL3; 5 = SV-g2-J10-C-pGL3; 6 = hPPARg2-CMV-Jx5AS-TK-pGL3; 7 = hPPARg2-CMV-Jx10AS-CMV-pGL3; 8 = hPPARg2-CMV-Jx15AS-CMV-pGL3; 9 = hPPARg2-CMV-Jx20AS-CMV-pGL3.

Figure 21 : Comparison in vitro of the ligands BRL49653 and RG12525. Mouse myoblasts (C2C12) are transfected with: (i) 10 ng of plasmid hPPARg2-CMV-Jx10AS-CMV-pGL3 whose expression cassette is presented in (**a**) and (ii) 10 ng of plasmid pRL-null. (**b**) The activity of the inducible promoter represents the luciferase activity of Photinus pyralis normalized using the activity of Renilla reniformis luciferase.

Figure 22 : Comparison of the different versions of the system inducible in vivo. C57BI/6 mice (6 mice per group) are injected bilaterally, in their cranial tibial, with, for each version of the system, the same number of moles of inducible expression cassettes. An electrotransfer is then applied on each muscle. The treated animals receive each day, by force-feeding, 30 mg/kg of BRL49653. Four days after the injection of DNA, the animals are sacrificed and the muscles are removed in order to measure the luciferase activity. 1 = pCMV-leadTK; 2 = pSG5-hPPARg2 + Jx10AS-CMV-pGL3; 3 = pSG5-hPPARg2g2 + Jx10AS-CMV-pGL3; 4 = hPPARg2-CMV-Jx10AS-CMV-pGL3.

Figure 23 : Comparison, in vivo, of various protocols for induction with BRL49653. C57BI/6 mice (6 mice per group) are injected bilaterally, in the cranial tibial,

with 10 µg of DNA containing 1 µg of plasmid hPPARg2-CMV-Jx10AS-CMV-pGL3 whose expression cassette is presented in (**a**). The activities obtained with the various induction protocols are assembled in the panel (**b**).

Figure 24 : Schematic representation of the plasmid pRDA02.

Figure 25 : Kinetics of induction obtained in vivo with the inducible system. (**A**) Ten C57BI/6 mice are injected bilaterally, in the cranial tibial, with a DNA mixture containing 3 mg of plasmid pRDA02 and 3 mg of plasmid pSG5-hPPARg2. An electrotransfer is then applied on each muscle. Four days, and then 39 days after the injection of DNA, the animals are treated, by force-feeding, with 30 mg/kg of BRL49653. At various times, blood samples are collected over heparin and the enzymatic activity of the secreted alkaline phosphatase (hSeAP) is measured in the plasma, using the Phospha-Light™ kit (Tropix, PE Biosystems, Foster City, CA). (**B**) C57BI/6 mice (2 groups of 10 mice) are injected bilaterally, in the cranial tibial, with a DNA mixture containing 3 mg of plasmid pRDA02 and 3 mg of plasmid pSG5-hPPARg2. An electrotransfer is then applied on each muscle. Four days after the injection of DNA, the animals receive, by force-feeding, either a single dose

of BRL49653 (30 mg/kg), or one dose per day (30 mg/kg) for 5 days. At various times, blood samples are collected over heparin, and the enzymatic activity of hSeAP is measured in the plasma, using the "Phospha-Light" kit (Tropix). The results presented (induction factors) correspond to the ratio between the hSeAP activity measured on the day of interest and that obtained on D4.

Figure 26: Comparison, *in vivo*, of different PPARg ligands, and study of the dose effect of one of them. C57BI/6 mice (5 mice per group) are injected bilaterally, in the cranial tibial, with a DNA mixture containing 5 mg of plasmid pRDA02 and 5 mg of plasmid pSG5-hPPARg2. An electrotransfer is then applied on each muscle. Six days (**A**) or 10 days (**B**) after the injection of DNA, the animals are treated, by force-feeding, either with different PPARg ligands (**A**; BRL49653, Actos™ (Takeda Pharmaceuticals) and Avandia™ (SmithKline Beecham)), or with various doses of BRL49653 (**B**). At various times, blood samples are collected over heparin, and the enzymatic activity of hSeAP is measured in the plasma, using the "Phospha-Light" kit (Tropix). The results presented (induction factors) correspond to the ratio between the hSeAP activity measured on the day of interest and that obtained on D6 (**A**) or D10 (**B**).

Figure 27: Schematic representation of the plasmid Jx10AS-CMV-VEGF$_A$165.

MATERIALS AND METHODS

The methods conventionally used in molecular biology, such as preparative extractions of plasmid DNA, caesium chloride gradient centrifugation of plasmid DNA, electrophoresis on agarose gels, purification of DNA fragments by electroelution, precipitation of plasmid DNA in saline medium with ethanol or isopropanol, transformation in Escherichia coli are well known to persons skilled in the art and are abundantly described in the literature (Sambrook et al., "Molecular Cloning, a Laboratory Manual", Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1989).

The plasmid pGL3-Basic, used for the clonings of the various promoter regions, as well as the plasmid pRL-null, are of commercial origin (Promega Corporation). The plasmids pSG5 (Stratagene), pBluescript II SK+ (Stratagene) and pSL301 (Invitrogen Corporation) are also of commercial origin. The constructions of the expression plasmids pSG5-hPPARg2 (Fajas L. et al., *J. Biol. Chem.*, **272** (1997) 18779-18789) and pSG5-hPPARa(Koz) (Gervois P. et al., *Mol.*

Endocrinol., **13** (1999) 400-409) have previously been described.

The construction of the plasmid pCMV-leadTK has also previously been described in patent application FR 98/120000 of 25/09/98, and in patent application US SN 60/123,298 (provisional application).

It is recalled that this plasmid is constructed in the following manner. The expression vector pCGN previously described by Tanaka et al. (*Cell*, **60** (1990) 375-386) contains the CMV promoter (-522/+72) fused with the "leader" of the HSV tk gene (+51/+101) upstream of a sequence encoding the haemagglutinin epitope. The plasmid pCGN (10 ng) was used as template for a PCR amplification. The primers which were used are the following:

- Primer 6718 (5'CCCGTTACATAACTTACGGTAAATGGCCCG3') (SEQ ID NO : 26), this primer hydridizes with the CMV promoter at position -522 (8 nucleotides downstream of the EcoRI site of pCGN).

- primer 6719 (5'**G**GGACGCGCTTCTACAAGGCGCTGGCCGAA3') (SEQ ID NO : 27), this primer hybridizes up to position 101 of the tk "leader". The first nucleotide G in bold is intended to restore the NcoI site of pGL3-Basic as will be explained below.

The PCR fragment thus obtained is purified and then phosphorylated with the aid of the T4 phage polynucleotide kinase (New England Biolabs). In parallel, the vector pGL3-Basic (Promega) was linearized with NcoI, purified and then treated with Klenow DNA polymerase (Boehringer Mannheim) so as to fill the NcoI site. This vector is then dephosphorylated with the aid of alkaline phosphatase (Boehringer Mannheim) and then used for the insertion of the phosphorylated PCR fragment. Thus, the guanosine (G) of the primer 6719 makes it possible to restore only the NcoI site when the CMV-tk leader fragment is oriented with the 5' part (primer 6718, position -522 of the CMV) downstream of the HindIII site of pGL3-Basic and its 3' end (primer 6719, tk leader) is ligated to the NcoI site of pGL3-Basic (first ATG of luciferase). The plasmid thus obtained is designated pCMV-leadTK.

The enzymatic amplification of DNA fragments by the PCR (polymerase chain reaction) technique may be carried out using a DNA thermal cycler™ (Perkin Elmer Cetus) according to the manufacturer's recommendations.

The electroporation of plasmid DNA into Escherichia coli cells may be carried out with the aid of an electroporator (Bio-Rad) according to the manufacturer's recommendations.

The verification of the nucleotide sequences may be carried out by the method developed by Sanger et al., (*Proc. Natl. Acad. Sci. USA,* **74** (1977) 5463-5467) using the kit distributed by Applied Biosystems according to the manufacturer's recommendations.

The murine myoblasts C2C12 are cultured in DMEM™ medium (Life Technologies Inc.) supplemented with 10% foetal calf serum (FCS). The cultures are carried out in an oven at 37°C, under a humid atmosphere and at a CO_2 partial pressure of 5%.

The tranfections are carried out in 24-well plates and each transfection is carried out three times. Twenty-four hours after the transfection, the cells are inoculated at 3×10^4 cells per well in DMEM™ medium. For each well, 500 ng of plasmid DNA (plasmids of interest and pBluescript II SK+ in order to adjust to 500 ng) are mixed with the cationic lipid RPR120535 B (WO 97/18185) in an amount of 6 nmol of lipid per µg of DNA in DMEM™ medium (20 µl final) comprising 150 mM NaCl and 50 mM bicarbonate. After 20 minutes at room temperature, the 20 µl of the DNA/lipid mixture are brought into contact with the cells, in the absence of FCS, for 2 hours, The culture medium is then supplemented with FCS or with ULTROSER™ (BioSepra Inc.) so as to obtain a final concentration of 10% or 2% respectively. The PPAR ligands, dissolved in DMSO, are added to the culture medium at the same time as the FCS

or the ULTROSER™. Forty-eight hours after the transfection, the culture medium is removed and the cells are rinsed twice with PBS (Life Technologies Inc.). The activity of the Photinus pyralis luciferase and the activity of the Renilla reniformis luciferase are then determined with the aid of the Dual-Luciferase Reporter Assay System™ kit (Promega Corporation) according to the supplier's recommendations.

The in vivo gene transfer experiments are carried out on 6-week-old C57BI/6 female mice. The animals are anaesthetized with 250 µl of a ketamine (Rhône Mérieux, 10 mg/ml final)/Xylazine (Bayer Pharma, 0.3 mg/ml final) mixture by the intraperitoneal route. An injection of a total quantity of 10 µg of DNA is then carried out into each cranial tibial muscle. Each leg is then subjected to an electric field (frequency of 1 Hz; 4 pulses of 20 ms at 250 V/cm). During the entire duration of the experiment, the animals receive each morning, by force-feeding, either 30 mg/kg of BRL49653 (SmithKline Beecham) in 1% carboxycellulose (weight/volume), or 1% carboxycellulose alone. Four days after the gene transfer, the animals are sacrificed and the muscles collected in PLB™ lysis buffer (Promega Corporation) in Lysing Matrix™ tubes (BIO 101, Inc.). The grinding of the muscles, which makes it possible to extract luciferase, is carried out with the aid of the FastPrep™ apparatus (BIO 101, Inc.)

for 25 seconds at 6.5 m/s. The activity of the Photinus pyralis luciferase is then determined with the aid of the Luciferase Assay System™ kit (Promega Corporation) according to the supplier's recommendations.

EXAMPLES

EXAMPLE 1 : Construction of promoters inducible by the PPARs and of expression plasmids containing them.

1.1. Plasmid FTKpGL3.

A DNA fragment, corresponding to part of the promoter of the TK gene of the type 1 herpes simplex virus (HSV-1), between positions -105 and +56 relative to the site of initiation of transcription, was amplified by PCR using the plasmid pBLCAT2 (Luckow B. and Schutz G., *Nucleic Acids Res.*, **15** (1987) 5490) as template and the oligonucleotides 5' CGA CTC TAG AAG ATC TTG CCC CGC CCA GCG 3' (SEQ ID NO:28) and 5' TCG CCA AGC TTC TCG TGA TCT GCG GCA 3' (SEQ ID NO:2) as primers. This fragment was digested with BglII and HindIII and was then cloned into the plasmid pGL3-Basic previously digested with BglII and HindIII in order to obtain the plasmid FTKpGL3. A schematic representation of this plasmid is presented in Figure 1.

1.2 Plasmids JxnS-TK-pGL3.

A DNA fragment, containing one or more (n) J sites of the promoter of the human ApoA-II gene, was amplified by PCR using the plasmid J3TKpGL3 (Vu-Dac N. et al., *J. Clin. Invest.*, **96** (1995) 741-750) as template and the oligonucleotides 3RDA37 (5' ACG TGT CGA CAC TAG TGG CTA GAG GAT CTC TAC CAG G 3'; SEQ ID NO:3) and 4RDA48 (5' CGA TGG TAC CCT CGA GCA ATG TGC TAG CGA GAT CCT TCA ACC TTT ACC 3'; SEQ ID NO:4) as primers. This fragment was digested with XhoI and SpeI and was then cloned into the plasmid FTKpGL3 previously digested with XhoI and NheI, in the direction of transcription of the minimal TK promoter (S), in order to obtain the plasmids Jx1S-TK-pGL3, Jx2S-TK-pGL3 and Jx3S-TK-pGL3, depending on the number of J sites present. A schematic representation of the plasmid Jx3S-TK-pGL3 is presented in Figure 2.

The DNA fragment, amplified by PCR using the plasmid J3TKpGL3 and the oligonucleotides 3RDA37 and 4RDA48 as primers, digested with XhoI and SpeI, was also cloned into the plasmid Jx3S-TK-pGL3 previously digested with XhoI and NheI, in order to obtain the plasmids Jx4S-TK-pGL3, Jx5S-TK-pGL3 and Jx6S-TK-pGL3, depending on the number of J sites present.

1.3. Plasmids JxnAS-TK-pGL3.

The plasmids JxnAS-TK-pGL3 differ from the plasmids JxnS-TK-pGL3 by the orientation of the J sites present in the inducible promotor. A DNA fragment containing one or more J sites of the promoter of the human ApoA-II gene, was amplified by PCR using the plasmid J3TKpGL3 as template and the oligonucleotides 3RDA37 and 4RDA48 as primers. This fragment was digested with SalI and NheI and was then cloned, in the opposite direction of transcription of the minimal TK promotor (AS), into the plasmid FTKpGL3 previously digested with XhoI and NheI in order to obtain the plasmids Jx1AS-TK-pGL3, Jx2AS-TK-pGL3 and Jx3AS-TK-pGL3, depending on the number of J sites present. A schematic representation of the plasmid Jx3AS-TK-pGL3 is presented in Figure 3.

The DNA fragment, amplified by PCR using the plasmid J3TKpGL3 and the oligonucleotides 3RDA37 and 4RDA48 as primers, digested with KpnI and SpeI, was also cloned in the antisense (AS) orientation into the plasmid Jx3AS-TK-pGL3 previously digested with KpnI and NheI in order to obtain the plasmids Jx4AS-TK-pGL3 and Jx5AS-TK-pGL3 depending on the number of J sites present.

1.4 Plasmids DR1xnS-TK-pGL3.

These plasmids contain, as PPAR response element (PPRE), a consensus sequence (AGGTCA A AGGTCA, SEQ ID NO:5) called consensus DR1. A DNA fragment, containing one or more consensus DR1 sites, was amplified by PCR using the oligonucleotides 1RDA69 (5' ACG TGT CGA CAC TAG TCA AAA CTA GGT CAA AGG TCA CGG AAA ACT AGG TCA AAG GTC ACG GAG AAC TAG 3'; SEQ ID NO:6) and 2RDA64 (5' CGA TGG TAC CCT CGA GCA ATG TGC TAG CCG TGA CCT TTG ACC TAG TTT TCC GTG ACC TTT GAC C 3'; SEQ ID NO:7) as primers. This fragment was digested with digested with XhoI and SpeI and was then cloned, in the sense orientation, into the plasmid FTKpGL3 previously digested with XhoI and NheI in order to obtain the plasmids DR1x2S-TK-pGL3 and DR1x3S-TK-pGL3, depending on the number of consensus DR1 sites present. A schematic representation of the plasmid DR1x3S-TK-pGL3 is presented in Figure 4.

The DNA fragment, amplified by PCR using the oligonucleotides 1RDA69 and 2RDA64 as primers, digested with XhoI and SpeI, was also cloned into the plasmid DR1x3S-TK-pGL3 previously digested with XhoI and NheI in order to obtain the plasmids DR1x5S-TK-pGL3, DR1x6S-TK-pGL3 and DR1x7S-TK-pGL3, depending on the number of consensus DR1 sites present.

1.5. Plasmids DR1xnAS-TK-pGL3.

The plasmids DR1xnAS-TK-pGL3 differ from the plasmids DR1xnS-TK-pGL3 by the orientation of the consensus DR1 sites present in the inducible promoter. A DNA fragment, containing one or more consensus DR1 sequences, was amplified by PCR using the oligonucleotides 1RDA69 and 2RDA64 as primers. This fragment was digested with SalI and NheI, and was then cloned, in the antisense orientation, into the plasmid FTKpGL3 previously digested with XhoI and NheI in order to obtain the plasmids DR1x2AS-TK-pGL3 and DR1x3AS-TK-pGL3, depending on the number of consensus DR1 sites present. A schematic representation of the plasmid DR1x3AS-TK-pGL3 is presented in Figure 5.

The DNA fragment, amplified by PCR using oligonucleotides 1RDA69 and 2RDA64 as primers, digested with KpnI and SPeI, was also cloned into the plasmid DR1x3AS-TK-pGL3 previously digested with KpnI and NheI in order to obtain the plasmids DR1x5AS-TK-pGL3 and DR1x6AS-TK-pGL3 depending on the number of consensus DR1 sites present.

EXAMPLE 2 : Specificity of the PPARs for different response elements.

2.1. System using hPPARg2.

The activity of the inducible promotors, using hPPARg2 as transcriptional regulator, was evaluated in transient tranfection in mouse myoblasts (Figure 6). The results show that, depending on the response element (PPRE) used, the induction by the hPPARg2 ligand (BRL49653) and the final activity after activation vary. The best results were obtained using J sites as PPRE. Furthermore, the orientation of the PPRE is also important. In the case of the J site, the AS orientation is more favourable (Panel c).

2.2. System using hPPARa.

The results obtained with hPPARa as transcriptional regulator are assembled in Figure 7. Unlike hPPARg2, it is the consensus DR1 which is the best PPRE for hPPARa (Panels d and e).

These results therefore show (1) the functionality of the plasmids of the invention and (2) that depending on the PPAR chosen in the inducible system, it is important to select the PPRE most appropriate for the transcriptional regulator. This choice may influence the induction factor due to the presence of the ligand but also the level of activity reached after induction. It is understood that other PPREs can be used in the system of the invention.

EXAMPLE 3: Construction of promoters inducible by the PPARs containing a minimum promoter other than that of HSV1-TK such as for example the hCMV-IE minimum promoter.

3.1. Construction of the plasmids containing the hCMV-IE minimum promoter.

A DNA fragment, containing the hCMV-IE minimum promoter (from position -54 to position +48 relative to the site of initiation of transcription), was amplified by PCR using the plasmid pCMVβ (Clontech) as template and the oligonucleotides 5RDA32 (5' ACG TAG ATC TCG GTA GGC GTG TAC GGT GGG AG 3'; SEQ ID NO:8) and 6RDA29 (5' ACG TAA GCT TCT ATG GAG GTC AAA ACA GC 3'; SEQ ID NO:9) as primers. This fragment was digested with HindIII and BglII and was then cloned into the plasmid FTKpGL3 previously digested with HindIII and BglII in order to obtain the plasmid FCMVpGL3.

The plasmid Jx5AS-TK-pGL3 was digested with BglII and NheI in order to isolate the BglII-NheI fragment of 179 bp containing 5 copies of the J site. This fragment was inserted into the plasmid FCMVpGL3 previously digested with BglII and NheI in order to give the plasmid Jx5AS-CMV-pGL3. A schematic

representation of the plasmid Jx5AS-CMV-pGL3 is presented in Figure 8.

The plasmid Jx5AS-CMV-pGL3 was digested with SphI and NheI in order to isolate the SphI-NheI fragment of 982 bp containing 5 copies of the J site, the hCMV-IE minimum promoter and the 5' part of the gene encoding luciferase. This fragment was inserted into the plasmid Jx5AS-CMV-pGL3 previously digested with SphI and SpeI in order to give the plasmid Jx10AS-CMV-pGL3. The plasmids Jx15AS-CMV-pGL3 and Jx20AS-CMV-pGL3 were also obtained by following the same strategy.

3.2. Activity of plasmids containing the hCMV-IE minimum promoter.

A comparison of the minimum promoters which can be used in the inducible system was made in transient transfection. The results, which are assembled in Figure 9, show that depending on the minimum promoter, the final activity after induction may vary by a factor of 2. These results show in particular that, under the conditions tested, the CMV promoter appears to give a higher activity. Of course other minimum promoters, such as promoters not containing a TATA box, may be used.

EXAMPLE 4 : Importance of the number of response elements present in the inducible promoters.

The optimization of the number of PPREs present in the inducible promoter was studied in transient transfection. The results, presented in Figure 10, show that the higher the number of copies of the PPRE, the greater the induction factor for the ligand and the activity induced. On the other hand, if this number is too high, both the induction factor and the induced activity decrease, this being regardless of the quantity of hPPARg2 present in the assay (Figure 11). The optimum number of PPRE appears to be between 10 and 15.

EXAMPLE 5: Construction of a transcriptional regulator highly inducible by the PPAR ligands.

5.1. Construction of a transcriptional regulator comprising two copies of the ligand-binding domain. Construction of the plasmid pSG5-hPPARg2g2.

A DNA fragment, noted A, containing the DNA region complementary to hPPARg2 encoding the C-terminal part of the F domain, was amplified by PCR using the plasmid pSG5-hPPARg2 as template and the oligonucleotides 20RDA21 (5' GGT TTG CTG AAT GTG AAG CCC 3'; SEQ ID NO:10) and 21RDA42 (5' AGT CTC TAG AGC

TAC GCG TAC AAG TCC TTG TAG ATC TCC TGC 3'; SEQ ID NO:11) as primers. A DNA fragment, noted B, containing the DNA region complementary to hPPARg2 encoding the E and F domains, was amplified by PCR using the plasmid pSG5-hPPARg2 as template and the oligonucleotides 22RDA32 (5' AGT CAC GCG TGG GCG ATC TTG ACA GGA AAG AC 3'; SEQ ID NO:12) and 23RDA21 (5' GCC TTT GAG TGA GCT GAT ACC 3'; SEQ ID NO:13) as primers. The A fragment, digested with SacI and MluI and the B fragment, digested with MluI and XbaI, were cloned together into the plasmid pSG5-hPPARg2 previously digested with SacI and XbaI in order to obtain the plasmid pSG5-hPPARg2g2. This plasmid, whose schematic representation is presented in Figure 12, contains a complementary DNA which encodes a transcriptional regulator (noted hPPARg2g2) comprising two copies of the E and F domains, that is to say two ligand-binding domains.

The complete sequence of PPARγ2γ2 is represented below (SEQ ID NO:24):

MGETLGDSPIDPESDSFTDTLSANISQEMTMVDTEMPFWPTNFGISSVDLSVMEDHSHSFDI
KPFTTVDFSSISTPHYEDIPFTRTDPVVADYKYDLKLQEYQSAIKVEPASPPYYSEKTQLYN
KPHEEPSNSLMAIECRVCGDKASGFHYGVHACEGCKGFFRRTIRLKLIYDRCDLNCRIHKKS
RNKCQYCRFQKCLAVGMSHNAIRFGRMPQAEKEKLLAEISSDIDQLNPESADLRALAKHLYD
SYIKSFPLTKAKARAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIF
QGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEIIYTMLASLMNKDGVLISEG
QGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPI
EDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPL
LQEIYKDLYAWAILTGKTTDKSPFVIYDMNSLMMGEDKIKFKHITPLQEQSKEVAIRIFQGC
QFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKYGVHEIIYTMLASLMNKDGVLISEGQGF
MTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDSDLAIFIAVIILSGDRPGLLNVKPIEDI
QDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQIVTEHVQLLQVIKKTETDMSLHPLLQE
IYKDLY

The sequence of the C-terminal part of PPARγ2γ2, comprising the E and F domains, is the following sequence SEQ ID NO:25:

MMGEDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTL
LKYGVHEIIYTMLASLMNKDGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALEL
DDSDLAIFIAVIILSGDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTD
LRQIVTEHVQLLQVIKKTETDMSLHPLLQEIYKDLYAWAILTGKTTDKSPFVIYDMNSLMMG
EDKIKFKHITPLQEQSKEVAIRIFQGCQFRSVEAVQEITEYAKSIPGFVNLDLNDQVTLLKY
GVHEIIYTMLASLMNKDGVLISEGQGFMTREFLKSLRKPFGDFMEPKFEFAVKFNALELDDS
DLAIFIAVIILSGDRPGLLNVKPIEDIQDNLLQALELQLKLNHPESSQLFAKLLQKMTDLRQ
IVTEHVQLLQVIKKTETDMSLHPLLQEIYKDLY

5.2. Activity of the plasmid pSG5-hPPARg2g2.

The results presented in Figure 13 show that if the induced activity is lower using hPPARg2g2 as transcriptional regulator (Figure 13 **a** and **b**), the induction factor for the ligand (Figure 13 **c**) is much higher with this regulator. The difference between the two transcriptional regulators is explained by the fact that for hPPARg2g2, the background noise of the system in the absence of ligand is low and remains low, regardless of the quantity of regulator present. On the other hand, the higher the increase in hPPARg2g2, the higher the induced activity, which is not the case for the system using hPPARg2 which appears to saturate.

The presence of a second ligand-binding domain (hPPARg2g2) therefore confers on the transcriptional regulator greater inducibility by the ligand.

EXAMPLE 6 : Increase in the final activity of the inducible promoters.

6.1. Construction of an inducible expression cassette comprising the intron of hEF1a. Construction of the plasmid Jx10AS-CMV-EF-pGL3.

A DNA fragment, containing the first intron of the gene encoding hEF1a (from position +16 to position +984 relative to the site of initiation of transcription; Genbank accession number: E02627), was amplified by PCR using the oligonucleotides 25RDA35 (5' AGT CAC TAG TAA GCT TTT TGC CGC CAG AAC ACA GG 3'; SEQ ID NO:14) and 26RDA36 (5' AGT CAC TAG TCC ATG GCT GCC CAG TGC CTC ACG ACC 3'; SEQ ID NO:15) as primers. This fragment was digested with HindIII and NcoI and was then cloned into the plasmid Jx10AS-CMV-pGL3 previously digested with HindIII and NcoI in order to obtain the plasmid Jx10AS-CMV-EF-pGL3. A schematic representation of the plasmid Jx10AS-CMV-EF-pGL3 is presented in Figure 14.

6.2. Activity of the plasmid Jx10AS-CMV-EF-pGL3.

With the aim of increasing the final activity of the system, an enhancer sequence, situated in the

first intron of the hEF1a gene, was cloned in the vicinity of the inducible promoter. The results presented in Figure 15 show that the presence of the enhancer region increases the induced activity of the system, this being regardless of the quantity of transcriptional regulator used.

EXAMPLE 7 : Construction of plasmids comprising both a cassette for expression of the transcriptional regulator and an inducible expression cassette.

7.1. Plasmid Jx5AS-TK-luc-hPPARg2.

The plasmid pSG5-hPPARa(Koz) was digested with MluI and ScaI in order to isolate the MluI-ScaI fragment of 1229 bp containing the 3' region of the DNA complementary to hPPARa. This fragment was inserted into the plasmid pSL301 previously digested with MluI and SmaI in order to give the plasmid pSL-3'hPPARa.

The plasmid pSG5-hPPARa(Koz) was digested with SalI and MluI in order to isolate the SalI-MluI fragment of 1406 bp containing the SV40 virus early promoter and the 5' region of the DNA complementary to hPPARa. This fragment was inserted into the plasmid pSL-3'hPPARa previously digested with XhoI and MluI in order to give the plasmid pSL-hPPARa.

The plasmid pSL-hPPARa was digested with SpeI and SalI in order to isolate the SpeI-SalI fragment of 2664 bp containing the SV40 virus early promoter and the DNA complementary to hPPARa. This fragment was inserted into the plasmid pBluescript II SK+ previously digested with SpeI and SalI in order to give the plasmid pBS-hPPARa.

The plasmid pSG5-hPPARg2 was digested with AvrII and SacI in order to isolate the AvrII-SacI fragment of 2070 bp, noted C, containing the 5' region of the DNA complementary to hPPARg2. A DNA fragment, noted D, containing the 3' region of the DNA complementary to hPPARg2, was amplified by PCR using the plasmid pSG5-hPPARg2 as template and the oligonucleotides 10RDA21 (5' CAG GTT TGC TGA ATG TGA AGC 3'; SEQ ID NO:16) and 11RDA40 (5' TGA CGT GTC GAC CTA GTA CAA GTC CTT GTA GAT CTC CTG C 3'; SEQ ID NO:17) as primers. The C fragment and the D fragment, digested with SacI and SalI, were cloned together into the plasmid pBS-hPPARa previously digested with AvrII and SalI in order to obtain the plasmid pBS-hPPARg2.

The plasmid Jx5AS-TK-pGL3 was digested with KpnI and SalI in order to isolate the KpnI-SalI fragment of 2324 bp containing the luc+ gene under the control of an inducible promoter. This fragment was inserted into the plasmid pBS-hPPARg2 previously digested with KpnI and SalI in order to give the

plasmid Jx5AS-TK-luc-hPPARg2. A schematic representation of the plasmid Jx5AS-TK-luc-hPPARg2 is presented in Figure 16.

7.2. Plasmid SV-g2-J10-C-pGL3.

The plasmid pBS-hPPARg2 was digested with NotI and SalI in order to isolate the NotI-SalI fragment of 2622 bp, noted E, containing the DNA complementary to hPPARg2 under the control of the SV40 early promoter. A DNA fragment, noted F, containing the SV40 virus polyadenylation site, was amplified by PCR using the plasmid FTK-pGL3 as template and the oligonucleotides 18RDA31 (5' AGT CGT CGA CGC TTC GAG CAG ACA TGA TAA G 3'; SEQ ID NO:18) and 19RDA35 (5' AGT CGC TAG CGA CGG ATC CTT ATC GAT TTT ACC AC 3'; SEQ ID NO:19) as primers. The E fragment and the F fragment, digested with SalI and NheI, were cloned together into the plasmid Jx10AS-CMV-pGL3 previously digested with NotI and NheI in order to obtain the plasmid SV-g2-J10-C-pGL3. A schematic representation of the plasmid SV-g2-J10-C-pGL3 is presented in Figure 17.

7.3. Plasmid hPPARg2-CMV-Jx5AS-TK-pGL3.

A DNA fragment, noted G, containing the DNA complementary to hPPARg2, was amplified by PCR using

the plasmid Jx5AS-TK-luc-hPPARg2 as template and the oligonucleotides 12RDA50 (5' GTC AGC TAG CCT ACT CGA GCC ACC ATG GGT GAA ACT CTG GGA GAT TCT CC 3'; SEQ ID NO:20) and 13RDA42 (5' TAC GGG GTA CCC AGA CAT GAT AAG ATA CAT TGA TGA GTT TGG 3'; SEQ ID NO:21) as primers. A DNA fragment, noted H, containing the hCMV-IE minimum promoter (from position -54 to position +48 relative to the site of initiation of transcription), was amplified by PCR using the plasmid pCMVβ as template and the oligonucleotides 14RDA33 (5' GTC AGC TAG CCG GTA GGC GTG TAC GGT GGG AGG 3'; SEQ ID NO:22) and 15RDA33 (5' TAC GCT CGA GCT TCT ATG GAG GTC AAA ACA GCG 3'; SEQ ID NO:23) as primers. The G fragment, digested with KpnI and XhoI and the H fragment, digested with XhoI and NheI, were cloned together into the plasmid Jx5AS-TK-pGL3 previously digested with KpnI and NheI in order to obtain the plasmid hPPARg2-CMV-Jx5AS-TK-pGL3. A schematic representation of the plasmid hPPARg2-CMV-Jx5AS-TK-pGL3 is presented in Figure 18.

7.4. Plasmids hPPARg2-CMV-JxnAS-CMV-pGL3.

The plasmid Jx5AS-CMV-pGL3 was digested with NheI and SphI in order to isolate the NheI-SphI fragment of 982 bp containing the 5' region of the luc+ gene under the control of an inducible promoter. This fragment was inserted into the plasmid hPPARg2-CMV-

Jx5AS-TK-pGL3 previously digested with SpeI and SphI in order to give the plasmid hPPARg2-CMV-Jx10AS-CMV-pGL3. A schematic representation of the plasmid hPPARg2-CMV-Jx10AS-CMV-pGL3 is presented in Figure 19.

The plasmid Jx5AS-CMV-pGL3 was digested with NheI and SphI in order to isolate the NheI-SphI fragment of 982 bp containing the 5' region of the luc+ gene under the control of an inducible promoter. This fragment was inserted into the plasmid hPPARg2-CMV-Jx10AS-CMV-pGL3 previously digested with SpeI and SphI in order to give the plasmid hPPARg2-CMV-Jx15AS-CMV-pGL3.

The plasmid Jx10AS-CMV-pGL3 was digested with NheI and SphI in order to isolate the NheI-SphI fragment of 1151 bp containing the 5' region of the luc+ gene under the control of an inducible promoter. This fragment was inserted into the plasmid hPPARg2-CMV-Jx10AS-CMV-pGL3 previously digested with SpeI and SphI in order to give the plasmid hPPARg2-CMV-Jx20AS-CMV-pGL3.

EXAMPLE 8 : Comparison of the different versions of the inducible system in vitro.

Figure 20 assembles the results obtained in vitro with various versions of the inducible system. These results show that the systems using two plasmids

(Figure 20, lines 1, 3 and 4) like the systems with only one plasmid (Figure 20, lines 2 and 5 to 9) are functional; that is to say that the presence of a PPARg ligand (here BRL49653) greatly increases the expression of the gene placed under the control of the inducible promoter. It is also observed that for some systems (Figure 20, lines 3 and 7 to 9), the induction factor for the ligand is greater than 30, and that for the system presented in Figure 20, line 4, the activity after induction is equal to that of a strong promoter such as that of the hCMV-IE promoter.

EXAMPLE 9 : Various PPAR ligands can activate the inducible system

9.1. System using hPPARg2.

The results presented in Figure 21 show that hPPARg ligands other than BRL49653, here RG12525 (RPR ligand for hPPARg), may be used to activate the inducible system. At a concentration of 100 µm, a treatment with RG12525 even leads to a higher induction than that obtained with BRL49653. Any other PPARg ligand may therefore be used as inducer of the system.

9.2. System using hPPARa.

In the same manner as for the system using hPPARg, a system using hPPARa as transcriptional regulator may be activated with the fibrates or WY-14,643 for example or any other hPPARa ligand.

EXAMPLE 10 : The inducible system may be activated in vivo, in the muscle.

Figure 22 assembles the results obtained in vivo, in the muscle, with different versions of the inducible system. The results show that for the three versions tested (Figure 22, lines 2 to 4), a treatment by force-feeding with a hPPARg ligand is capable of greatly increasing, in the muscle, the activity of the inducible promoters. The induction factors are: ×14 for the Figure 22 line 2 version, ×8 for the Figure 22 line 3 version, and ×24 for the Figure 22 line 4 version. Furthermore, for one of the versions (Figure 22, line 2), the activity obtained in the animals treated with BRL49653 is of the order of that of a strong promoter such as the hCMV-IE promoter.

The results, presented in Figure 23, also show that a single dose of ligand can induce the system, whether this dose is taken before or after the gene transfer. This experiment also shows that a dose

which is two times smaller than that normally used makes it possible to obtain the same induction factor.

The system, using a PPAR nuclear receptor as transcriptional regulator, is therefore functional in vivo and may be induced by the oral administration of a PPAR ligand.

EXAMPLE 11: Construction of a plasmid allowing the inducible expression of a gene whose product is secreted.

11.1 Construction of the plasmid pRDA02

The plasmid Jx10AS-CMV-pGL3 was digested with HindIII and MluI in order to isolate the HindIII-MluI fragment of 459 bp. This fragment was inserted into the plasmid pXL3010 (Bettan M. et al., *Anal. Biochem.*, **271** (1999) 187-189) previously digested with HindIII and MluI in order to give the plasmic pRDA02. This plasmid contains the DNA complementary to the gene encoding the secreted form of human placental alkaline phosphatase (hSeAP) whose expression is under the control of a promoter inducible by the system using the PPARs as transcriptional regulator. A schematic representation of the plasmid pRDA02 is presented in Figure 24.

EXAMPLE 12: The inducible system makes it possible to regulate, in vivo, the plasma concentration of a secreted protein.

The results presented in Figure 25 show that, by using the inducible system, it is possible to regulate, over time, the plasma concentration of a protein secreted from the muscle, this being with a simple oral administration of a PPAR ligand. The plasma concentration of hSeAP is increased by a factor of 18 (Figure 25A) two days after the administration of ligand, and then returns to its base level one week later. Between the 21st and 39th day, an immune response directed against hSeAP of human origin is observed and results in a decrease in the plasma concentration of this protein. Despite this immune response, it is possible to carry out a second induction cycle (Figure 25A).

As shown in Figure 25B, the inducible system also makes it possible, by daily administrations of ligand, to maintain the plasma level of hSeAP at a high level for a period equal to the duration of the treatment.

EXAMPLE 13: Various PPAR ligands can activate the inducible system in vivo, this being in a dose-dependent manner.

BRL49653, in its commercial form for the treatment of type II diabetes (Avandia™, SmithKline Beecham) and pioglitazone, in its commercial form for this same treatment (Actos™, Takeda Pharmaceuticals) can also activate the inducible system (Figure 26A). Figure 26B also shows that the induction factor is directly correlated with the dose of ligand used.

The system, using a PPAR nuclear receptor as transcriptional regulator, therefore makes it possible to control, very precisely, the plasma level of a secreted protein. Furthermore, this regulation may be obtained using various PPAR ligands.

EXAMPLE 14: Construction of a plasmid allowing the inducible expression of a gene whose product is an angiogenic factor.

14.1 Construction of the plasmid Jx10AS-CMV-$VEGF_A165$.

The human VEGF165 reading frame was cloned by reverse transcription and PCR from total RNA of human placenta (Clontech) (Houck et al. *Mol. Endocrinol.* **12**

(1991) 1806-1814) and then inserted into a plasmid pBluescript (Stratagene) containing the CMV E/P promoter from position -522 to +72 and the SV40 late polyA, in order to give the plasmid pXL3218. The latter was then digested with HindIII and BsrGI in order to isolate the HindIII-BsrGI fragment A of 482 bp. The plasmid pXL3218 was also digested with BsrGI and BamHI in order to isolate the BsrGI-BamHI fragment B of 390 bp. Fragments A and B were inserted into the plasmid Jx10AS-CMV-pGL3 previously digested with HindIII and BamHI in order to give the plasmid Jx10AS-CMV-$VEGF_A165$. This plasmid contains the DNA complementary to the gene encoding $VEGF_A165$ whose expression is under the control of a promoter inducible by the system using PPARs as transcriptional regulator. A schematic representation of the plasmid Jx10AS-CMV-$VEGF_A165$ is presented in Figure 27.

This plasmid can be used, for example, to control, over time, the angiogenic activity of VEGF for therapeutic purposes.

AMENDED CLAIMS

[received by the international office on 3 January 2001 (03.01.01);

original claims 1-30 replaced by new claims 1-33 (4 pages)]

1. Composition comprising:

(a) a first element comprising a nucleic acid of interest under the control of an inducible promoter comprising a PPAR response element and a minimal transcriptional promoter, and

(b) a second element comprising a nucleic acid encoding a PPAR under the control of a transcriptional promoter,

for their use simultaneously, separately or spaced out over time.

2. Composition according to Claim 1, characterized in that it comprises in addition:

(c) a ligand for PPAR,

for a use simultaneously, separately or spaced out over time.

3. Composition according to Claim 1 or 2, characterized in that the elements (a) and (b) are carried by distinct genetic constructs.

4. Composition according to Claim 1 or 2, characterized in that the elements (a) and (b) are assembled in the same genetic construct.

5. Composition according to Claim 3 or 4, characterized in that the genetic construct is a plasmid or viral vector.

6. Composition according to Claim 5, characterized in that the viral vector is an adeno-associated virus (AAV).

7. Composition according to one of Claims 1 to 6, characterized in that the PPAR response element comprises one or more PPAR-binding sites.

8. Composition according to Claim 7, characterized in that the PPAR response element comprises one or more sites having the sequence SEQ ID NO:1 or functional variants of this sequence.

9. Composition according to Claim 7, characterized in that the PPAR response element comprises one or more sites having the sequence SEQ ID NO:5 or functional variants of this sequence.

10. Composition according to Claims 7 to 9, characterized in that the response element comprises up to 30 binding sites, preferably from 3 to 20, more preferably from 5 to 15.

11. Composition according to one of Claims 1 to 10, characterized in that the minimal promoter is a promoter of a cellular or viral gene deleted for the region(s) not essential for transcriptional activity.

12. Composition according to one of Claims 1 to 11, characterized in that the inducible promoter comprises, in addition, an enhancer region.

13. Composition according to one of Claims 1 to 12, characterized in that the minimal promoter and the PPAR response element are in the same orientation.

14. Composition according to one of Claims 1 to 12, characterized in that the minimal promoter and the PPAR response element are in the opposite orientation.

15. Composition according to one of Claims 1 to 14, characterized in that the nucleic acid encoding a PPAR encodes a PPARα or a PPARγ.

16. Composition according to one of Claims 1 to 15, characterized in that the nucleic acid encoding a PPAR encodes a modified PPAR comprising several ligand-binding sites.

17. Composition according to one of Claims 1 to 16, characterized in that it comprises, in addition, an element (d) comprising a nucleic acid encoding an RXR under the control of a transcriptional promoter.

18. Vector comprising an element (a) and an element (b) according to Claim 1.

19. Vector according to Claim 18, characterized in that the elements (a) and (b) are in the opposite orientation.

20. Vector according to Claim 18 or 19, characterized in that the inducible promoter of the element (a) and the transcriptional promoter of the element (b) are assembled in the vector to form a regulable bidirectional promoter.

21. Vector according to Claim 20, characterized in that it comprises, in the 5'→3' direction, a first nucleic acid encoding a PPAR, a first minimal transcriptional promoter controlling the expression of the said first nucleic acid, one or more PPAR response elements, a second minimal transcriptional promoter and, under the control of the said second minimal transcriptional promoter, a second nucleic acid encoding a product of interest.

22. Vector according to one of Claims 18 to 21, characterized in that it comprises, in addition, an element (d) according to Claim 17.

23. Use of a composition according to one of Claims 1 to 17 or of a vector according to one of Claims 18 to 22 for expressing a nucleic acid of interest in a cell ex vivo or in vitro.

24. Use of a composition according to one of Claims 1 to 17 or of a vector according to one of Claims 18 to 22 for the preparation of a product intended for expressing a nucleic acid of interest in a cell in vivo.

25. Method for the regulated expression of a nucleic acid in a cell, in vitro or ex vivo comprising bringing the said cell into contact with a composition according to one of Claims 1 to 17 or a vector according to one of Claims 18 to 22.

26. Method according to Claim 25, characterized in that it is a mammalian, preferably human cell.

27. Method according to Claim 26, characterized in that it is a muscle cell.

28. Method for regulating the expression of a nucleic acid in vivo comprising the administration of a composition according to one of Claims 1 to 17 or of a vector according to one of Claims 18 to 22.

29. Cell modified by bringing into contact with a composition according to one of Claims 1 to 17 or a vector according to one of Claims 18 to 22.

30. Modified PPAR comprising several ligand-binding sites.

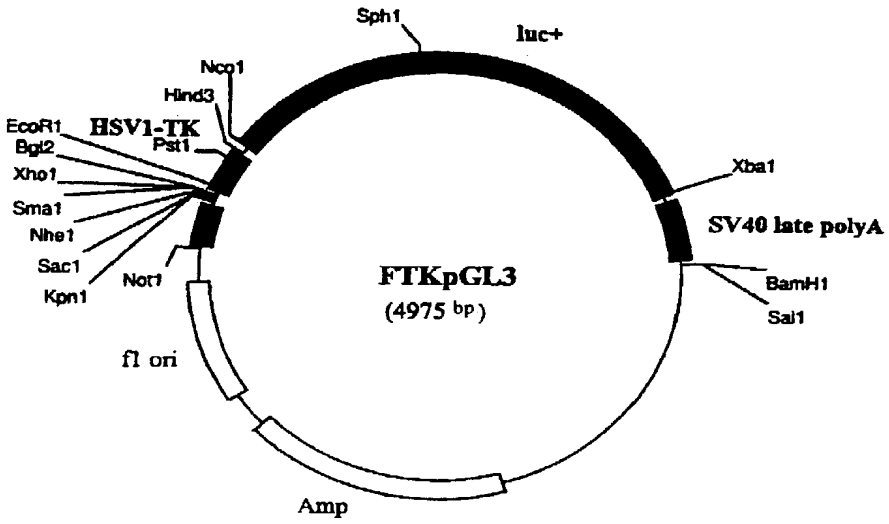
31. Nucleic acid encoding a PPAR according to Claim 30.

32. Method for identifying PPAR ligands, comprising the bringing of a cell according to Claim 29 into contact with a test molecule and the detection of the expression of the nucleic acid of interest.

33. Method for identifying PPAR ligands in vivo, characterized in that there is administered a composition according to one of Claims 1 to 17 or a vector according to one of Claims 18 to 22 as well as a test molecule, and in that the expression of the nucleic acid of interest is detected.

ABSTRACT OF THE DISCLOSURE

Provided herein are novel methods and compositions for the pharmacological regulation of the expression of transgenes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luc+
Nco1
Hind3
EcoR1
HSV1-TK
Pst1
Bgl2
Xho1
Sma1
Nhe1
Sac1
Kpn1
Not1
Xba1
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FTKpGL3
(4975 bp)
BamH1
Sal1
f1 ori
Amp

FIGURE 1

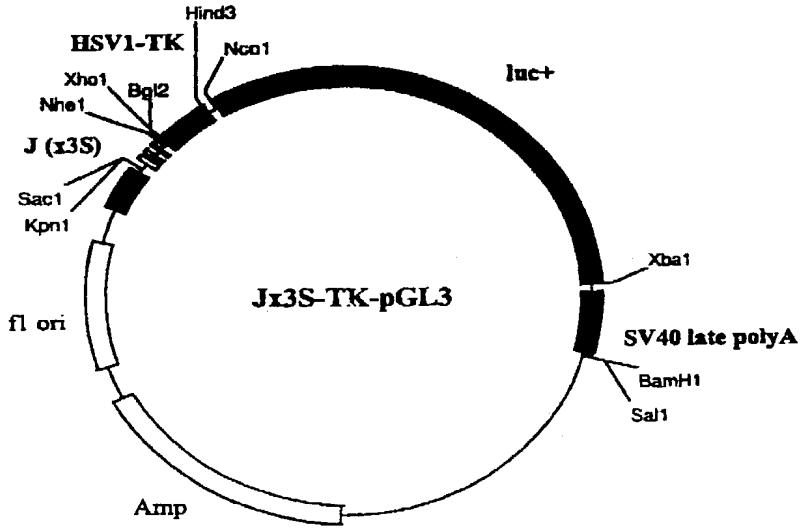

Hind3
HSV1-TK
Nco1
Xho1
Bgl2
Nhe1
J (x3S)
Sac1
Kpn1
luc+
Xba1
Jx3S-TK-pGL3
fl ori
SV40 late polyA
BamH1
Sal1
Amp

FIGURE 2

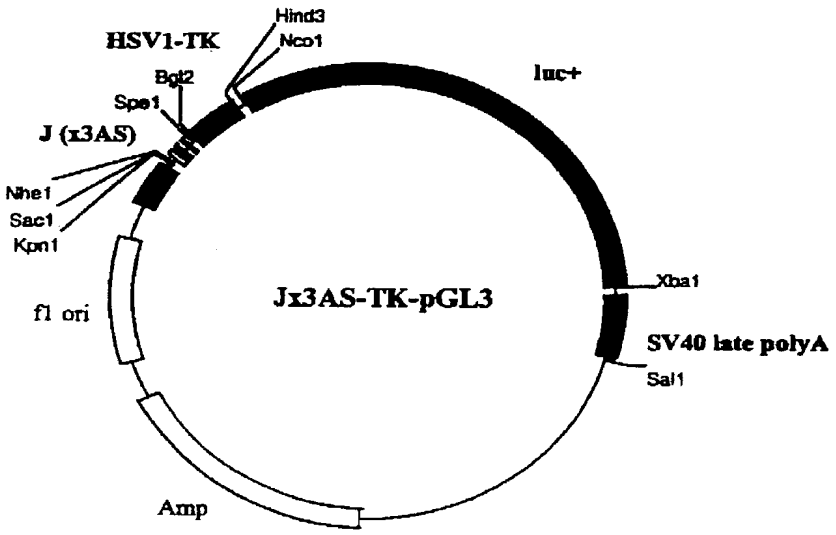

Hind3
HSV1-TK
Nco1
Bgl2
luc+
Spe1
J (x3AS)
Nhe1
Sac1
Kpn1
Jx3AS-TK-pGL3
Xba1
f1 ori
SV40 late polyA
Sal1
Amp

FIGURE 3

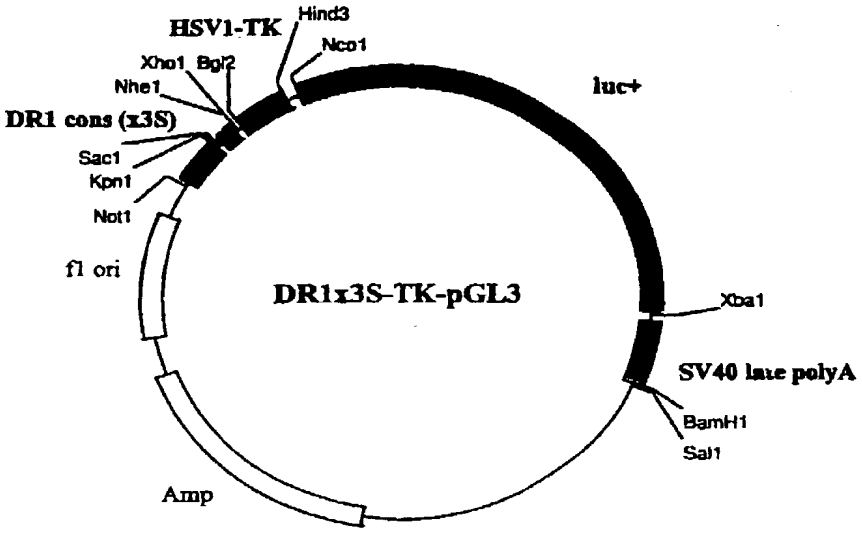

HSV1-TK
Hind3
Nco1
Xho1
Bgl2
Nhe1
DR1 cons (x3S)
Sac1
Kpn1
Not1
f1 ori
luc+
DR1x3S-TK-pGL3
Xba1
SV40 late polyA
BamH1
Sal1
Amp

FIGURE 4

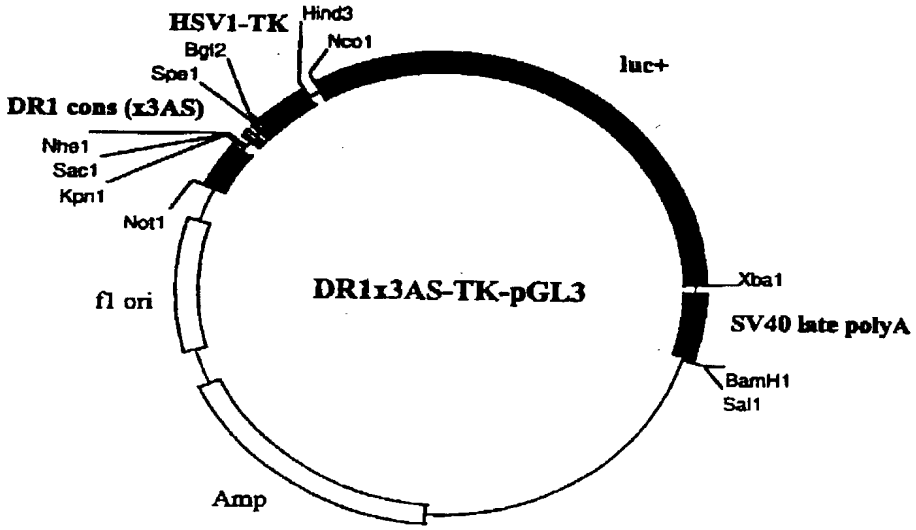

HSV1-TK
Hind3
Nco1
Bgl2
Spe1
DR1 cons (x3AS)
Nhe1
Sac1
Kpn1
Not1
luc+
fl ori
DR1x3AS-TK-pGL3
Xba1
SV40 late polyA
BamH1
Sal1
Amp

FIGURE 5

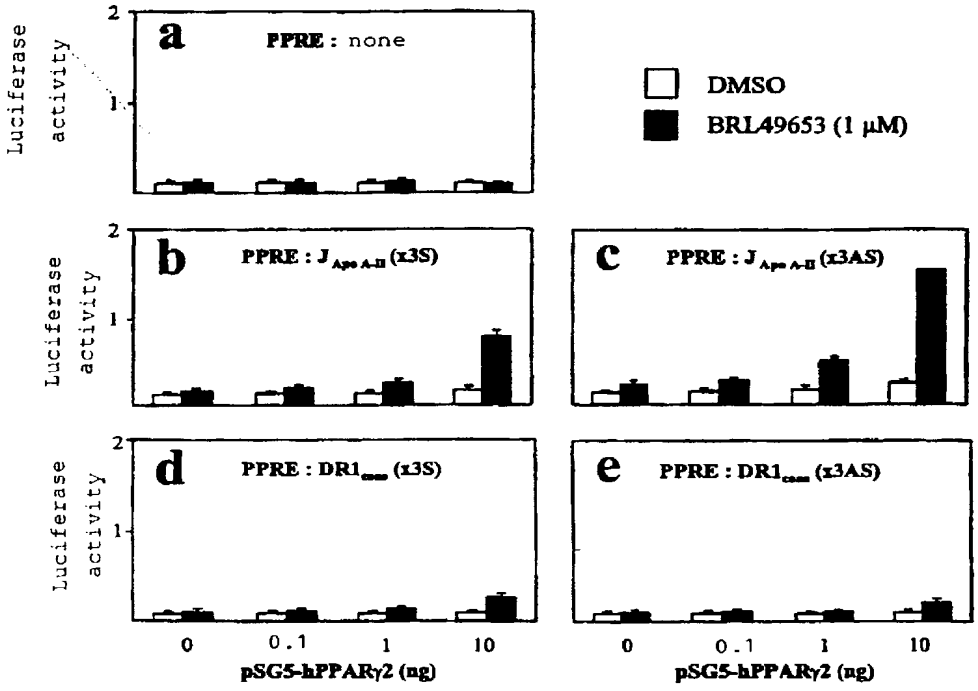

a
PPRE : none
DMSO
BRL49653 (1 µM)
b
PPRE : J Apo A-II (x3S)
c
PPRE : J Apo A-II (x3AS)
d
PPRE : DR1 con (x3S)
e
PPRE : DR1 con (x3AS)
Luciferase activity
2
1
0
0.1
1
10
pSG5-hPPARγ2 (ng)

FIGURE 6

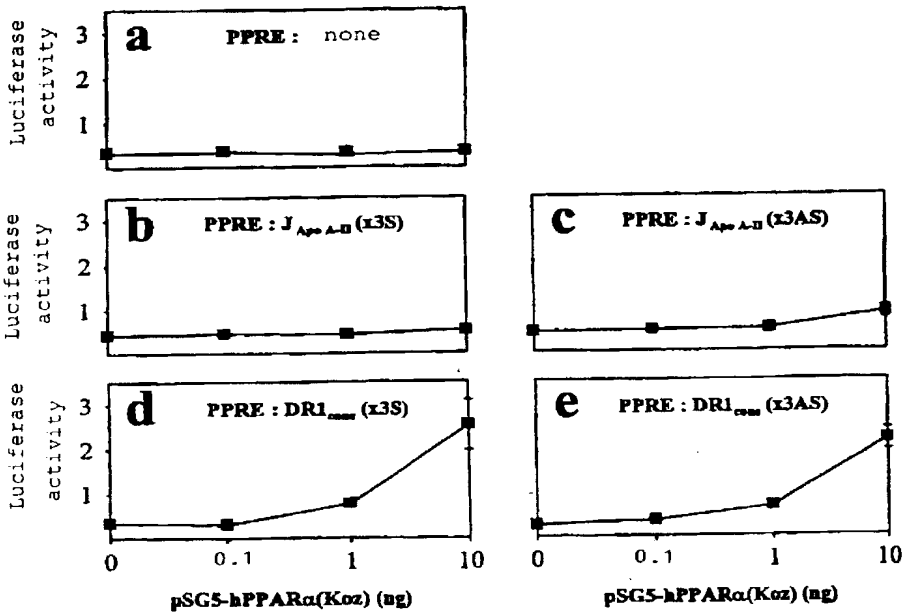

a
PPRE : none
b
PPRE : J Apo A-II (x3S)
c
PPRE : J Apo A-II (x3AS)
d
PPRE : DR1 cons (x3S)
e
PPRE : DR1 cons (x3AS)
Luciferase activity
3
2
1
0
0.1
1
10
pSG5-hPPARα(Koz) (ng)

FIGURE 7

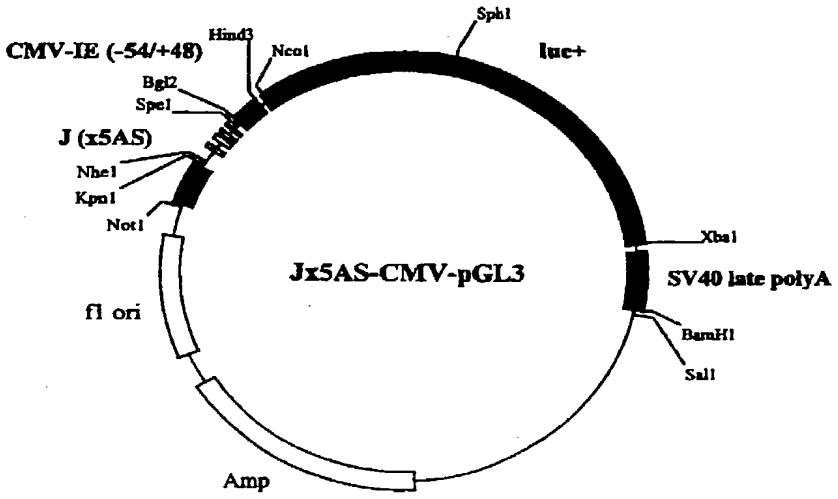

CMV-IE (-54/+48)
Hind3
Nco1
Sph1
luc+
Bgl2
Spe1
J (x5AS)
Nhe1
Kpn1
Not1
Jx5AS-CMV-pGL3
Xba1
SV40 late polyA
BamH1
Sal1
f1 ori
Amp

FIGURE 8

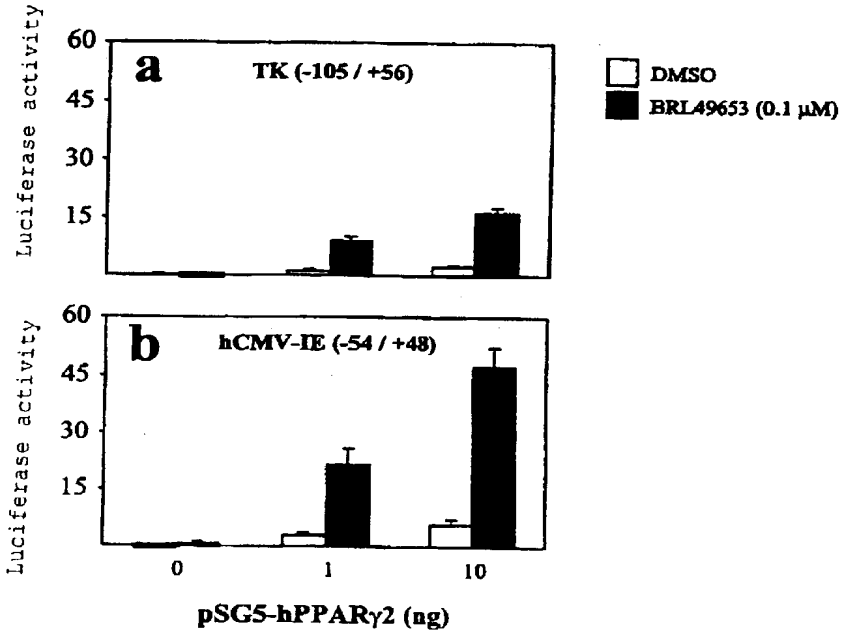

Luciferase activity
60
45
30
15
a
TK (-105 / +56)
Luciferase activity
60
45
30
15
b
hCMV-IE (-54 / +48)
0
1
10
pSG5-hPPARγ2 (ng)
DMSO
BRL49653 (0.1 µM)

FIGURE 9

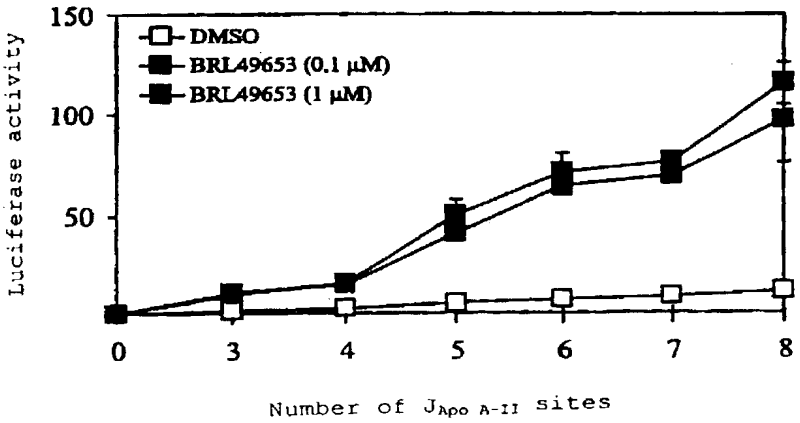

150
100
50
0
3
4
5
6
7
8
DMSO
BRL49653 (0.1 µM)
BRL49653 (1 µM)
Luciferase activity
Number of JApo A-II sites

FIGURE 10

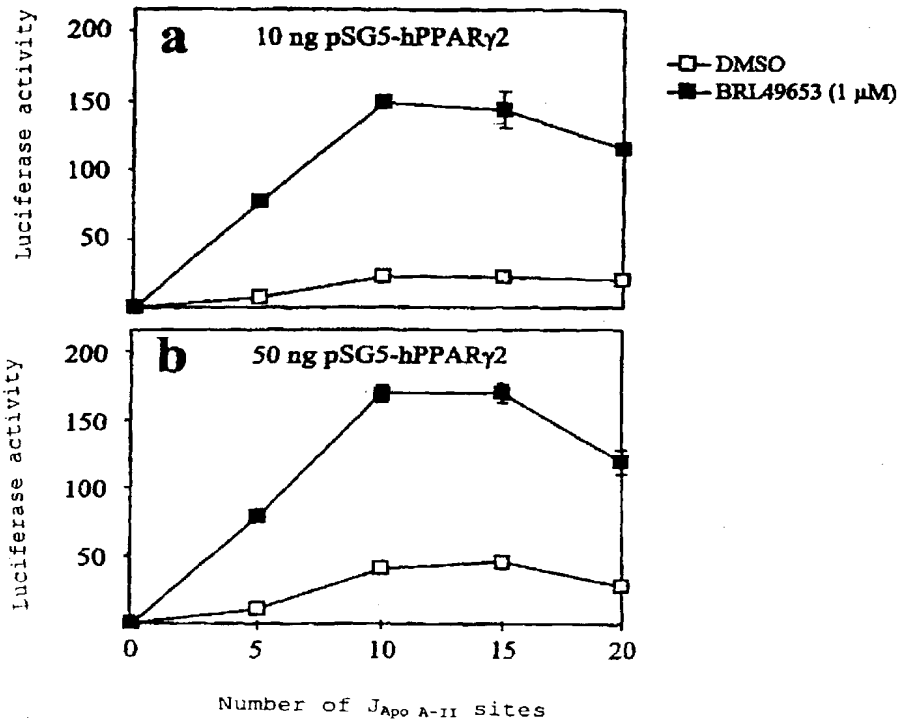

a
10 ng pSG5-hPPARγ2
b
50 ng pSG5-hPPARγ2
Luciferase activity
200
150
100
50
0
5
10
15
20
DMSO
BRL49653 (1 µM)
Number of J Apo A-II sites

FIGURE 11

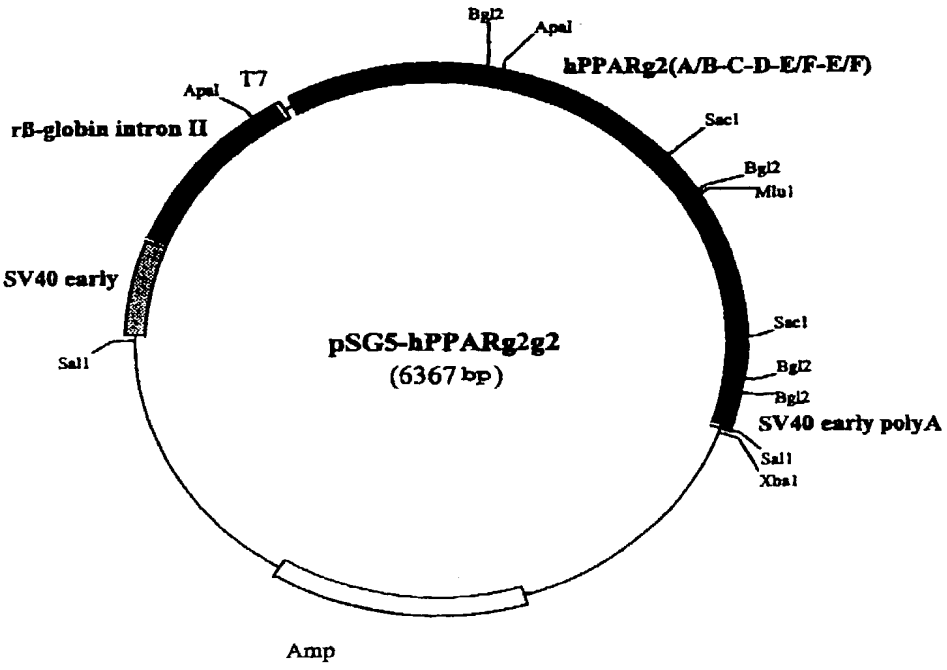

Bgl2
ApaI
hPPARg2(A/B-C-D-E/F-E/F)
SacI
Bgl2
MluI
SacI
Bgl2
Bgl2
SV40 early polyA
SalI
XbaI
Amp
SalI
SV40 early
rß-globin intron II
ApaI
T7
pSG5-hPPARg2g2
(6367 bp)

FIGURE 12

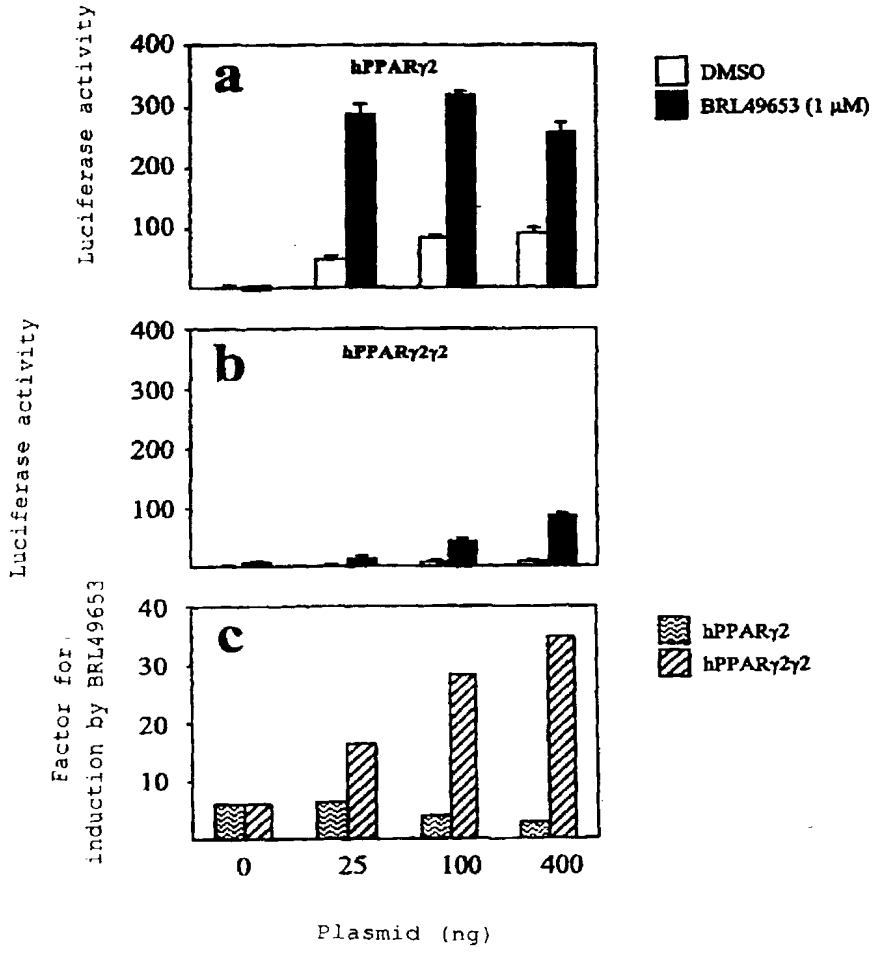

Luciferase activity
400
300
200
100
a
hPPARγ2
DMSO
BRL49653 (1 µM)
Luciferase activity
400
300
200
100
b
hPPARγ2γ2
Factor for induction by BRL49653
40
30
20
10
c
hPPARγ2
hPPARγ2γ2
0
25
100
400
Plasmid (ng)

FIGURE 13

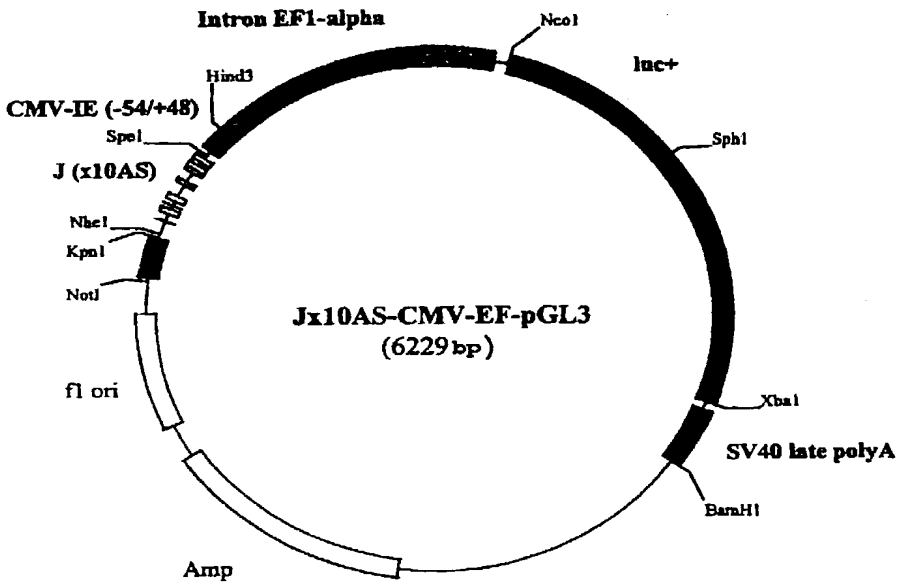

Intron EF1-alpha
NcoI
luc+
Hind3
CMV-IE (-54/+48)
SpeI
SphI
J (x10AS)
NheI
KpnI
NotI
Jx10AS-CMV-EF-pGL3
(6229 bp)
f1 ori
XbaI
SV40 late polyA
BamHI
Amp

FIGURE 14

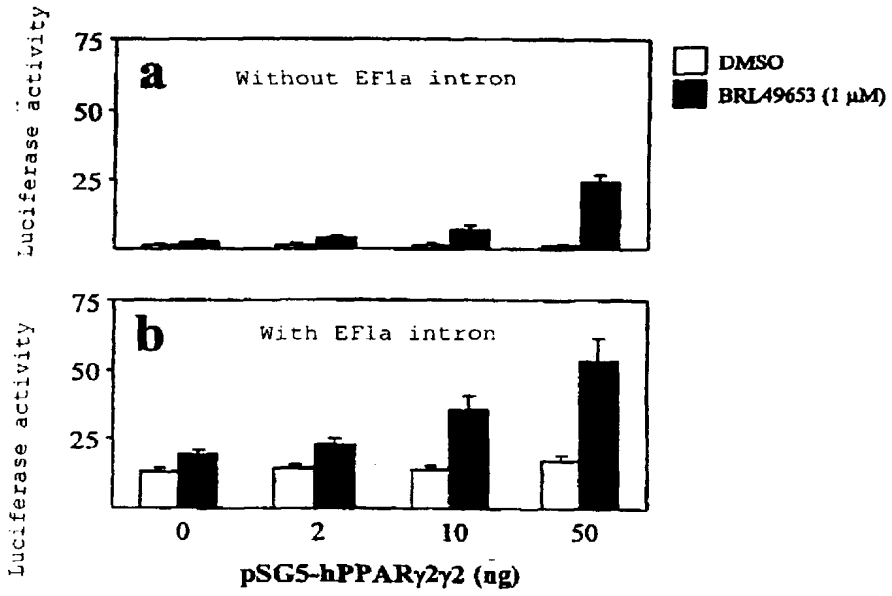

Luciferase activity
75
50
25
a
Without EF1a intron
DMSO
BRL49653 (1 µM)
Luciferase activity
75
50
25
b
With EF1a intron
0
2
10
50
pSG5-hPPARγ2γ2 (ng)

FIGURE 15

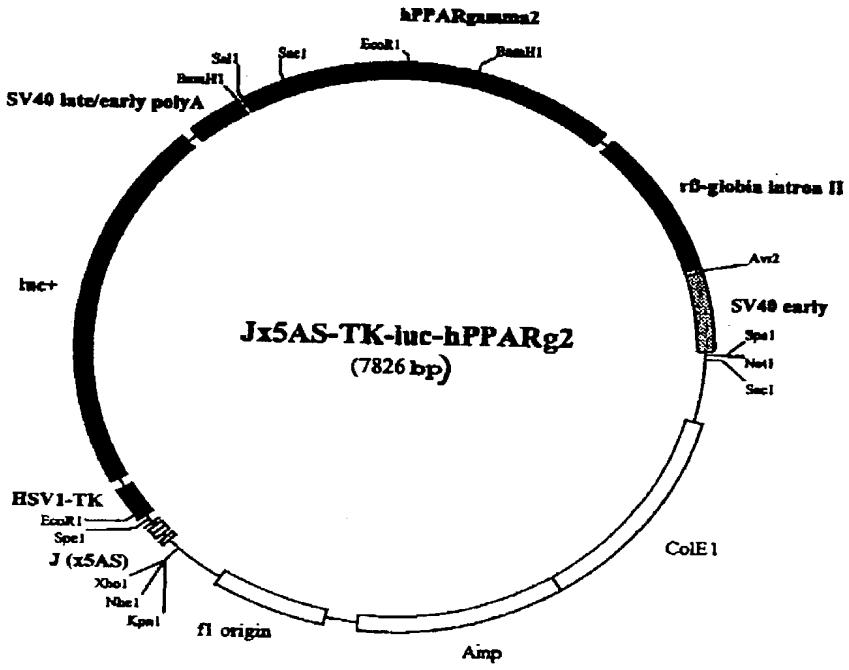

hPPARgamma2
EcoR1
BamH1
Sac1
Sal1
BamH1
SV40 late/early polyA
rß-globin intron II
Avr2
SV40 early
Spe1
Not1
Sac1
luc+
Jx5AS-TK-luc-hPPARg2
(7826 bp)
HSV1-TK
EcoR1
Spe1
J (x5AS)
Xho1
Nhe1
Kpn1
f1 origin
ColE1
Amp

FIGURE 16

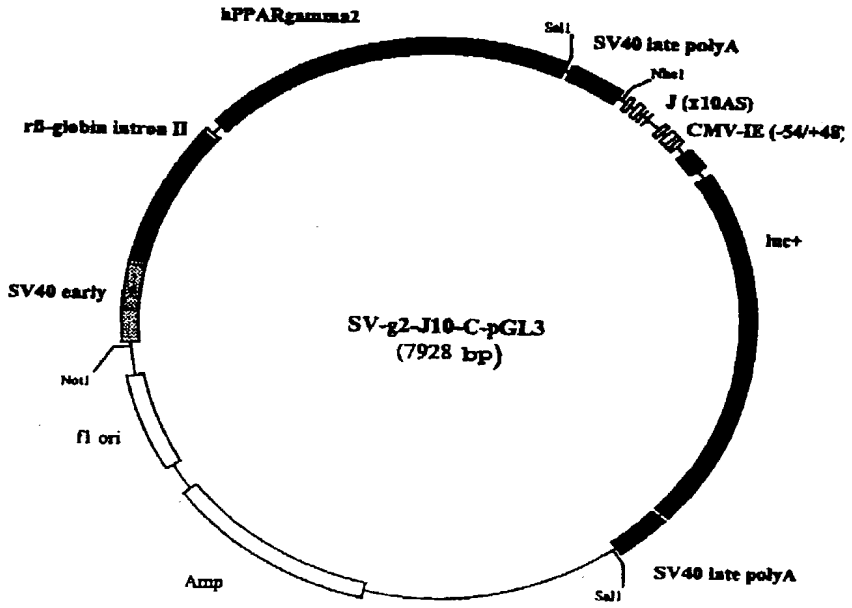

hPPARgamma2
SalI
SV40 late polyA
NheI
J (x10AS)
CMV-IE (-54/+48)
luc+
SV40 late polyA
SalI
Amp
f1 ori
NotI
SV40 early
rß-globin intron II
SV-g2-J10-C-pGL3
(7928 bp)

FIGURE 17

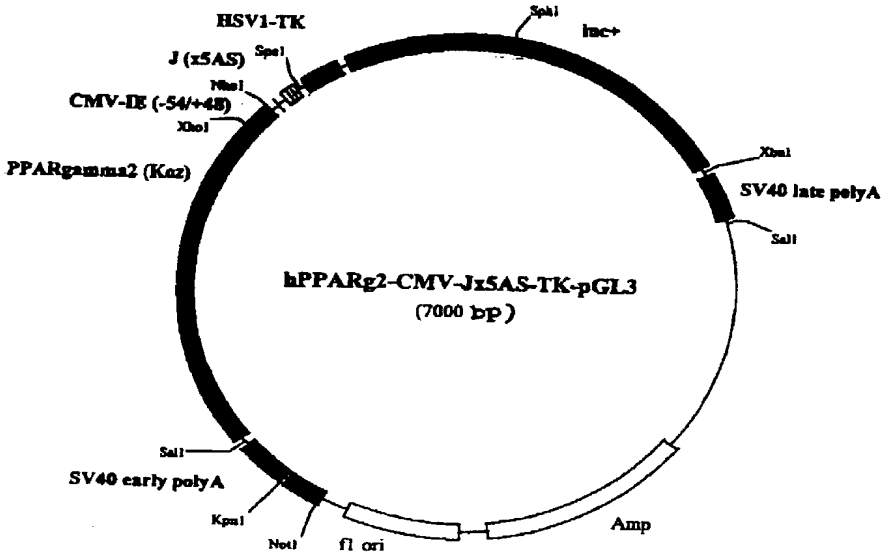

HSV1-TK
J (x5AS)
CMV-IE (-54/+48)
PPARgamma2 (Koz)
SpeI
NheI
XhoI
SpeI
Inc+
XbaI
SV40 late polyA
SalI
hPPARg2-CMV-Jx5AS-TK-pGL3
(7000 bp)
SalI
SV40 early polyA
KpnI
NotI
f1 ori
Amp

FIGURE 18

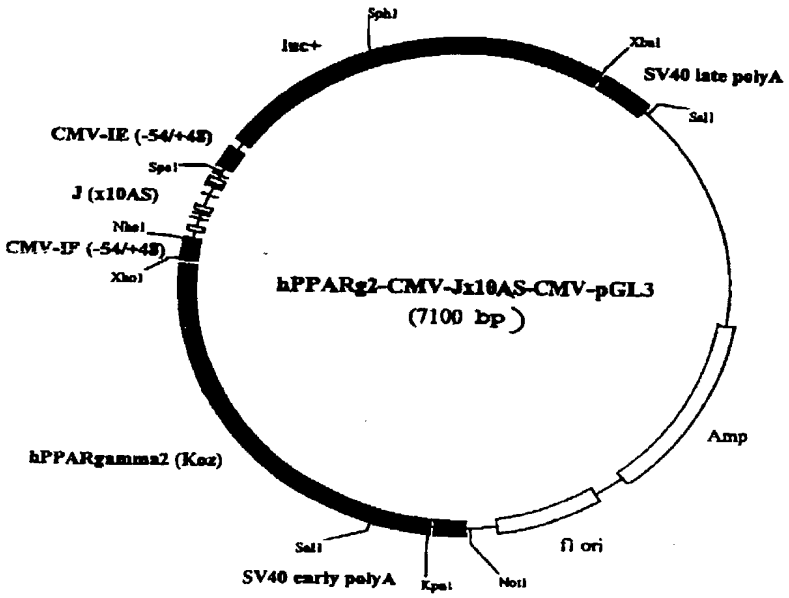

SphI
Luc+
XbaI
SV40 late polyA
SalI
CMV-IE (-54/+45)
SpeI
J (x10AS)
NheI
CMV-IF (-54/+45)
XhoI
hPPARg2-CMV-Jx10AS-CMV-pGL3
(7100 bp)
Amp
hPPARgamma2 (Koz)
SalI
SV40 early polyA
KpnI
NotI
f1 ori

FIGURE 19

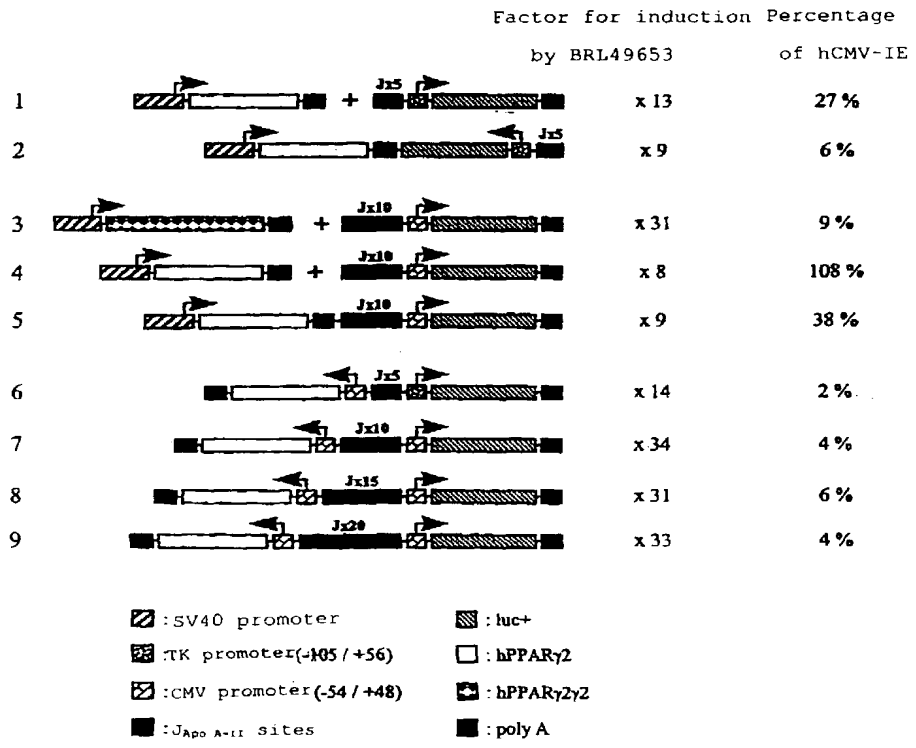

Factor for induction by BRL49653
Percentage of hCMV-IE
1
x 13
27 %
2
x 9
6 %
3
x 31
9 %
4
x 8
108 %
5
x 9
38 %
6
x 14
2 %
7
x 34
4 %
8
x 31
6 %
9
x 33
4 %
Jx5
Jx10
Jx15
Jx20
: SV40 promoter
: TK promoter (-105 / +56)
: CMV promoter (-54 / +48)
: JApo A-II sites
: luc+
: hPPARγ2
: hPPARγ2γ2
: poly A

FIGURE 20

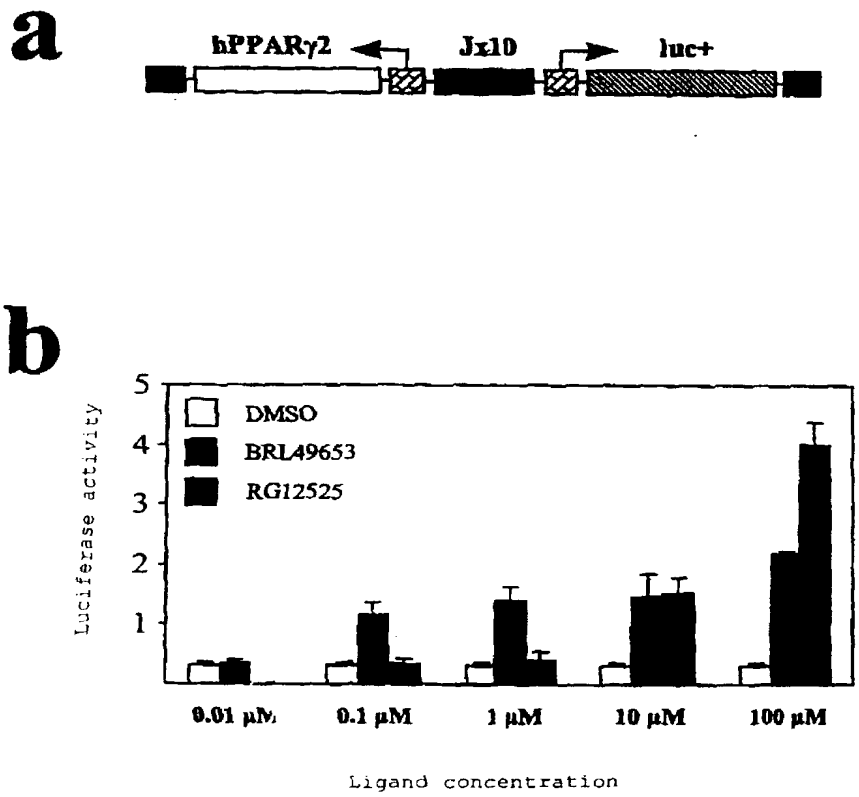

a
hPPARγ2
Jx10
luc+
b
Luciferase activity
5
4
3
2
1
DMSO
BRL49653
RG12525
0.01 µM
0.1 µM
1 µM
10 µM
100 µM
Ligand concentration

FIGURE 21

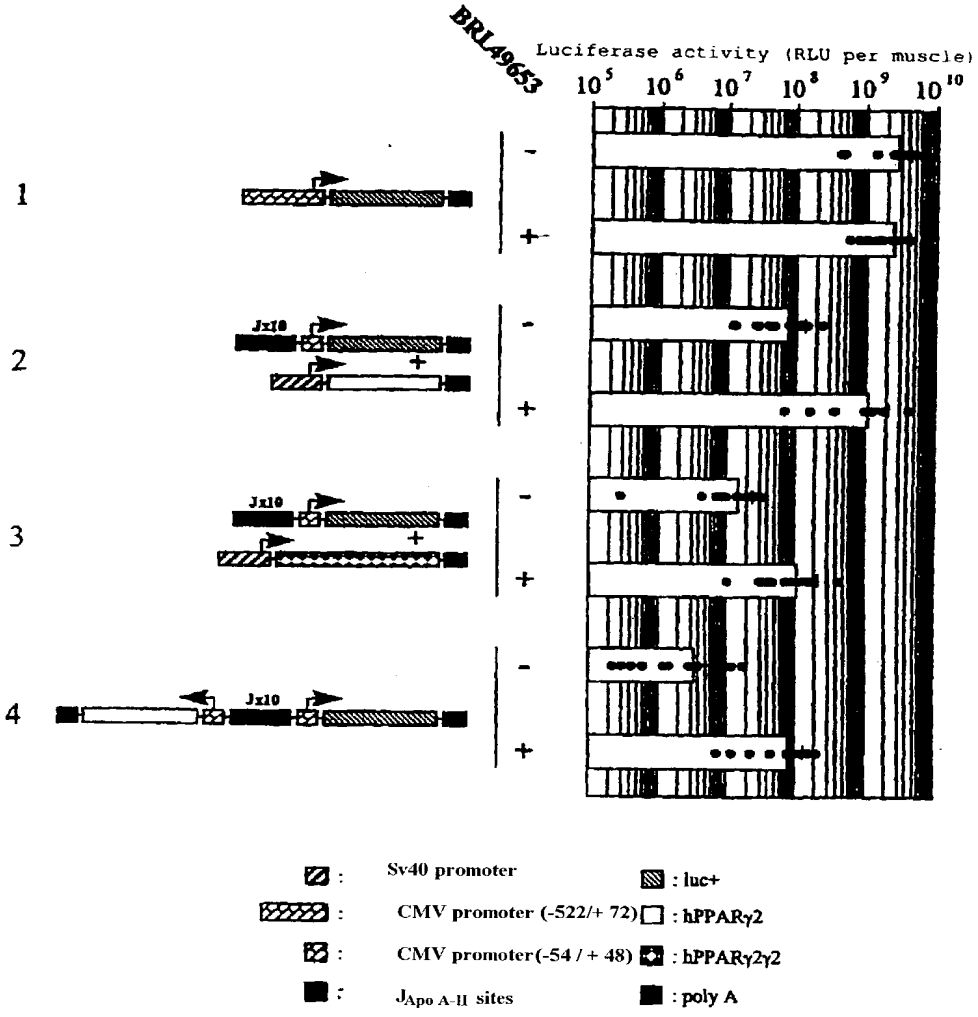

BRL49653
Luciferase activity (RLU per muscle)
10^5 10^6 10^7 10^8 10^9 10^{10}
1
2
3
4
Jx10
- +
: Sv40 promoter
: luc+
: CMV promoter (-522/+ 72)
: hPPARγ2
: CMV promoter (-54 / + 48)
: hPPARγ2γ2
: $J_{Apo\ A\text{-}II}$ sites
: poly A

FIGURE 22


a
Jx10

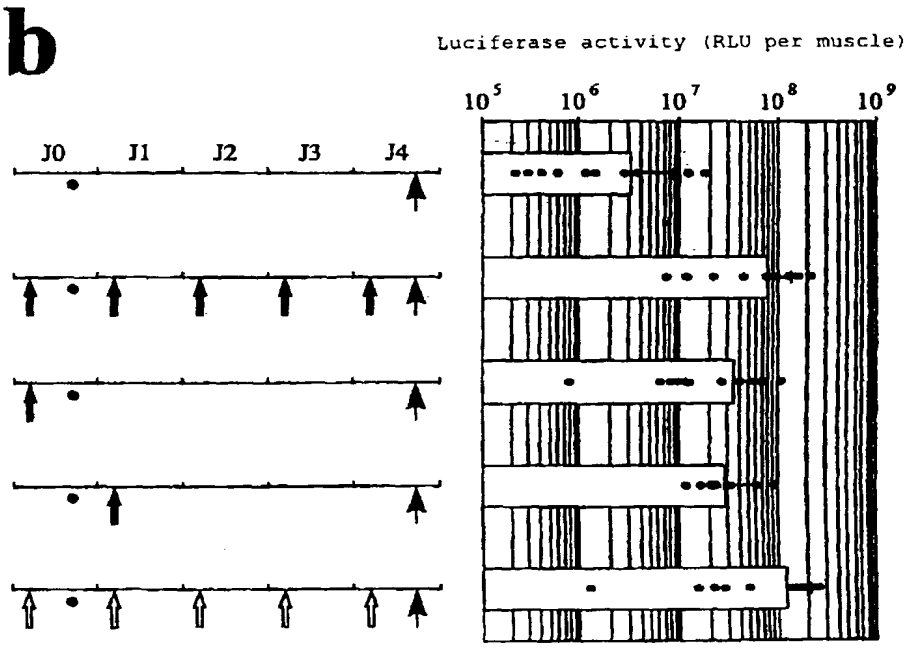

b
Luciferase activity (RLU per muscle)
10^5
10^6
10^7
10^8
10^9
J0
J1
J2
J3
J4
: Administration of BRL49653 (30 mg/kg)
: Administration of BRL49653 (15 mg/kg)
: injection of the plasmid and electrotransfer
: sacrificing of the animals

FIGURE 23

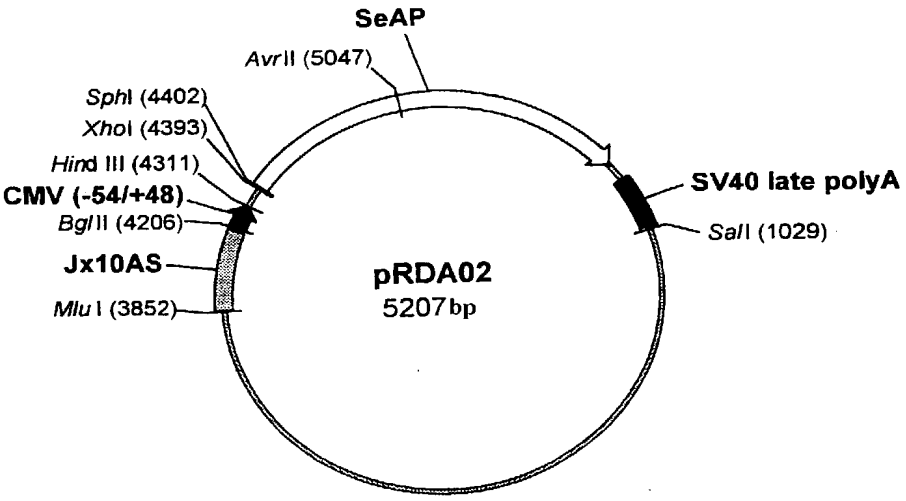
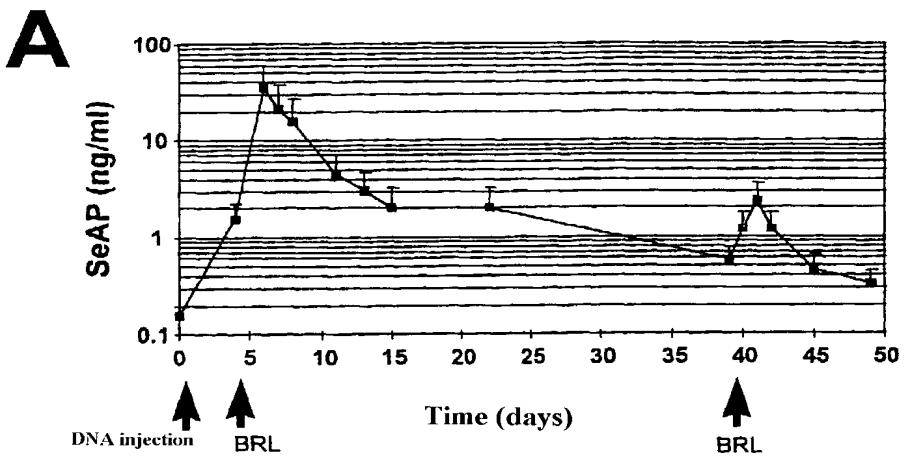

SeAP
AvrII (5047)
SphI (4402)
XhoI (4393)
Hind III (4311)
CMV (-54/+48)
BglII (4206)
Jx10AS
MluI (3852)
pRDA02
5207bp
SV40 late polyA
SalI (1029)

FIGURE 24


A
SeAP (ng/ml)
100
10
1
0.1
0
5
10
15
20
25
30
35
40
45
50
Time (days)
DNA injection
BRL
BRL

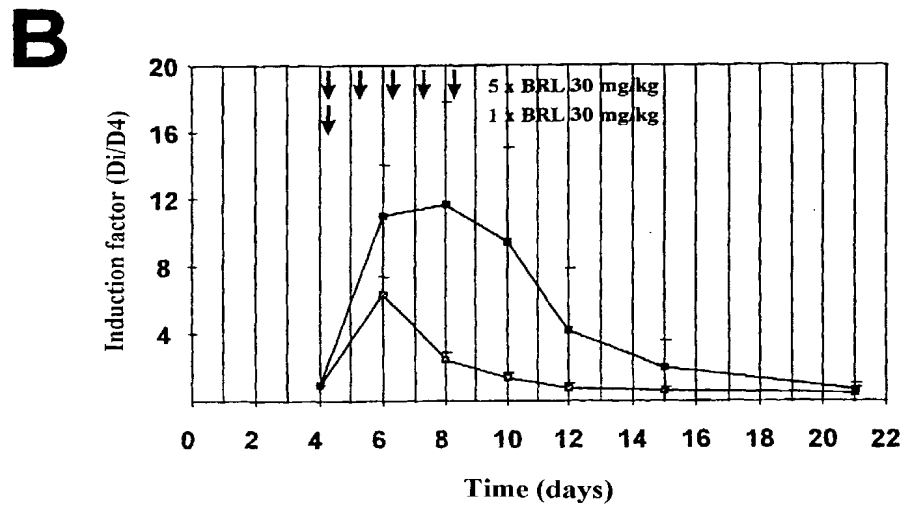
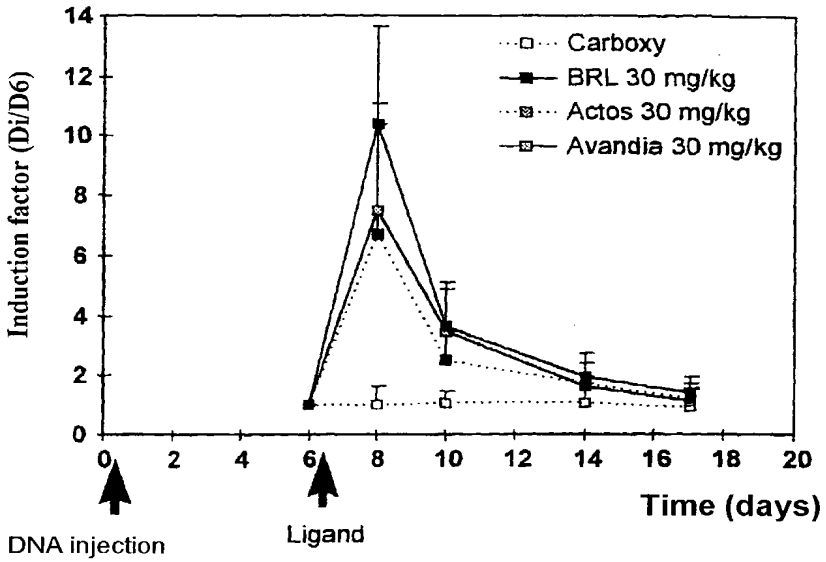

B
Induction factor (Di/D4)
20
16
12
8
4
5 x BRL 30 mg/kg
1 x BRL 30 mg/kg
0
2
4
6
8
10
12
14
16
18
20
22
Time (days)

FIGURE 25

A


Induction factor (Di/D6)
Carboxy
BRL 30 mg/kg
Actos 30 mg/kg
Avandia 30 mg/kg
Time (days)
DNA injection
Ligand

B

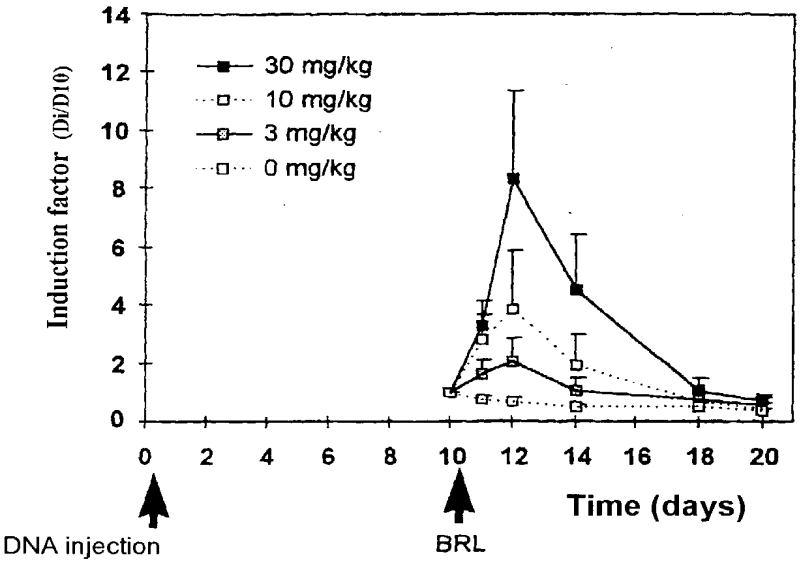

Induction factor (Di/D10)
30 mg/kg
10 mg/kg
3 mg/kg
0 mg/kg
Time (days)
DNA injection
BRL

FIGURE 26

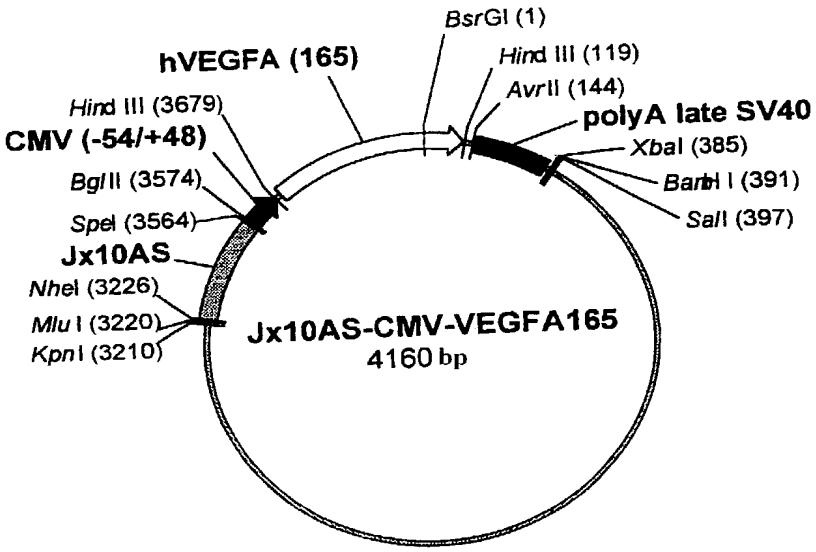

BsrGI (1)
hVEGFA (165)
Hind III (119)
AvrII (144)
Hind III (3679)
polyA late SV40
CMV (-54/+48)
XbaI (385)
BglII (3574)
BamH I (391)
SpeI (3564)
SalI (397)
Jx10AS
NheI (3226)
MluI (3220)
Jx10AS-CMV-VEGFA165
4160 bp
KpnI (3210)

FIGURE 27

UNITED STATES OF AMERICA

DECLARATION AND POWER OF ATTORNEY FOR PATENT APPLICATION

As a below named inventor(s), I/We hereby declare that:

My/Our residence(s), post office address(es) and citizenship(s) are as stated below my name(s).
I/We verily believe I am/we are the original, first and sole/joint inventor(s) of the subject matter which is claimed and for which a patent is sought on the invention entitled:

SYSTEM FOR REGULATION OF EXPRESSION USING PPAR NUCLEAR RECEPTORS

and the specification of which (check one)
- ☐ is attached hereto (Attorney Docket No.**ST99021 US PCT**)
- ☒ was filed on **December 18, 2001** as U.S. Application Number **10/018,729** and was amended on (if applicable).
- ☐ was described and claimed in PCT Int'l Application Number filed on and as amended under PCT Article 19 on (if any).

I/We hereby state that I/We have reviewed and understand the contents of the above identified specification, including the claims as amended by any amendment referred to above.
I/We acknowledge the duty to disclose to the U.S. Patent and Trademark Office all information known to me/us to be material to patentability as defined in 37 C.F.R. 1.56.

☒ I/We hereby claim foreign priority benefits under Title 35, United States Code §119(a)-(d) or 365 (b) of any foreign application(s) for patent or inventor's certificate, or 365(a) of any PCT international application which designated at least one other country other than the United States of America, listed below and having a filing date before that of the application on which priority is claimed. I/We have also identified below any foreign application for patent or inventor's certificate having a filing date before that of the application on which priority is claimed:

	Number	Country	Day/Month/Year Filed
Foreign Priority:	**99 07957**	**France**	**22 June 1999**
Prior Foreign Appln(s):			

☒ I/We hereby claim the benefit under Title 35, United States Code §119(e) of any United States Provisional application(s) listed below:

Number	Filing Date
60/149,721	**August 20, 1999**

☒ I/We hereby claim the benefit under Title 35, United States Code §120 or 365(c) of any United States application(s) or international application designating the United States listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States application in the manner provided by the first paragraph of Title 35, United States Code §112, I/We acknowledge the duty to disclose information which is material to patentability as defined in Title 37, Code of Federal Regulations §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application.

Application Serial No.	Filing Date	Status (Patented, Pending)
PCT/FR00/01744	**June 22, 2000**	**Completed**

I/We hereby appoint the attorneys and/or agents associated with the Customer No.(s) provided below as my/our attorneys and/or agents with full power to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

Customer No.: 005487

I/We hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Title 18, United States code §1001, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Inventors(s): ☐ Additional names and signatures are attached.

1-00

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Signature: JOEL CROUZET

Date: 3/03/2002

Country of Citizenship: **France**

Residence: **Sceaux, France** FRX
(City and State/City and Country only)

P. O. Address: **12 rue Michel Voisin**
92330 Sceaux, France

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3. Full name: **STAELS, Bart**

Signature:

Date: 18-2-2002

Country of Citizenship: **Belgium**

Residence: **Kraainem, Belgium** BEX
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P. O. Address: **155 Ave d'Huart**
1950 Kraainem, Belgium

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4. Full name: **MAHFOUDI, Abderrahim**

Signature:

Date: 3-14-2002

Country of Citizenship: **France**

Residence: **Marolles en Brie, France** FRX
(City and State/City and Country only)

P. O. Address: **41 rue des Bergers**
94440 Marolles en Brie, France

5. Full name:

Signature:

Date:

Country of Citizenship:

Residence:
(City and State/City and Country only)

P. O. Address:

6. Full name:

Signature:

Date:

Country of Citizenship:

Residence:
(City and State/City and Country only)

P. O. Address:

7. Full name:

Signature:

Date:

Country of Citizenship:

Residence:
(City and State/City and Country only)

P. O. Address:

8. Full name:

Signature:

Date:

Country of Citizenship:

Residence:
(City and State/City and Country only)

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531 Rec'd PCT/PT 18 DEC 2001
WO 00/78986 PCT/FR00/01744

SEQUENCE LISTING

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